



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 49/00, C07H 21/02, 21/04, A01N 43/04		A1	(11) International Publication Number: WO 98/37919 (43) International Publication Date: 3 September 1998 (03.09.98)
(21) International Application Number: PCT/US98/03678 (22) International Filing Date: 25 February 1998 (25.02.98)		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 60/039,405 28 February 1997 (28.02.97) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; 214 Technology Innovation Center, Iowa City, IA 52242-5000 (US).			
(72) Inventors: SCHWARTZ, David, A.; 4810 Rapid Creek Road N.E., Iowa City, IA 52240 (US). KRIEG, Arthur, M.; 890 Park Place, Iowa City, IA 52246 (US).			
(74) Agent: HAILE, Lisa, A.; Fish & Richardson P.C., Suite 1400, 4225 Executive Square, La Jolla, CA 92037 (US).			
(54) Title: USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CpG DINUCLEOTIDE IN THE TREATMENT OF LPS-ASSOCIATED DISORDERS			
(57) Abstract			
<p>The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide affect immune responses in a subject. These nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide can be used to treat pulmonary disorders having an immunologic component, such as a response to inhaled lipopolysaccharide. The invention provides methods of treating subjects who have or are at risk of having these pulmonary disorders, and methods of altering the immunological components of the pulmonary disorders. The invention also provides pharmaceutical compositions for treating pulmonary disorders that have an immunologic component.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

USE OF NUCLEIC ACIDS CONTAINING UNMETHYLATED CpG
DINUCLEOTIDE IN THE TREATMENT OF LPS-ASSOCIATED
DISORDERS

5

FIELD OF THE INVENTION

This invention relates to generally to pulmonary disorders, and specifically to the use of oligonucleotides having at least one unmethylated CpG dinucleotide (CpG ODN) in the treatment of such disorders.

10

BACKGROUND OF THE INVENTION

Endotoxin is one of the primary mediators of inflammation released by Gram negative organisms and is an important cause of environmentally induced airway disease, such as ARDS. Inhaled endotoxin can cause airflow obstruction in previously unexposed subjects. Inhaled endotoxin is strongly associated with the development of acute 15 decrements in airflow among cotton workers, wine confinement workers, and poultry workers. The concentration of endotoxin in the bioaerosol appears to be the most important occupational exposure associated with the development and progression of airway disease in agricultural workers (Schwartz, D.A., *et al.*, *Am. J. Respir. Crit. Care Med.* 152:603-8, 1995).

20 In addition to being related to several occupational pulmonary diseases, exposure to endotoxin and to its purified derivative lipopolysaccharide (LPS) is also associated with severe asthma. The concentration of endotoxin in the domestic environment adversely affects asthmatics, with higher concentrations of ambient endotoxin associated with greater degrees of airflow obstruction. In addition, asthmatic individuals develop airflow 25 obstruction at lower concentrations of inhaled endotoxin than normal controls. Exposure-response studies have shown that inhaled lipopolysaccharide (LPS) produces recruitment of neutrophils, activation of macrophages with production and release of pro-

- 2 -

inflammatory cytokines, and damage to airway epithelia in a dose-dependent manner. These studies indicate that endotoxin is an important cause of airway disease among exposed individuals.

The acute respiratory distress syndrome (ARDS) is a condition characterized by acute

5 hypoxemia respiratory failure due to pulmonary edema (reviewed in Honing, E.G., and Ingram, R.H., Jr., in: Harrison's Principles of Internal Medicine, 14th Edition, A.S. Fauci, *et al.* (eds.), McGraw-Hill, New York, pp. 1483-1486, 1998; and Goodman, R.B., *et al.*, *Am J. Respir. Crit. Care Med.* 154:602-11, 1996). ARDS represents a spectrum of responses to acute lung injury (ALI); these response occur as complications of a more

10 widespread systemic response to acute inflammation or injury. ALI develops rapidly after a predisposing condition triggers a systemic inflammatory response and is most strongly associated with conditions that produced direct alveolar injury or direct injury via the pulmonary capillary bed, such as aspiration, diffuse infection, toxic inhalation, direct injury to the alveolar epithelium, or sepsis syndrome. ALI is the consequence of

15 unregulated over-expression of usual systemic inflammatory responses to infection and/or injury. Injury involves the alveolar epithelium and the pulmonary capillary endothelium, and results in a complex cascade of events. Injury is produced by cellular events associated with neutrophils, macrophages, monocytes, and lymphocytes producing various cytokines, in turn producing cellular activation, chemotaxis, and adhesion.

20 Gram-negative infections are a major cause of morbidity and mortality, especially in hospitalized and immunocompromised patients. (Duma, *Am. J. of Med.*, 78 (Suppl. 6A):154-164, 1985; and Kreger *et al.*, *Am. J. Med.*, 68:344-355, 1980). Although available antibiotics are generally effective in inhibiting growth of Gram-negative bacteria, they do not neutralize the pathophysiological effects associated with endotoxins.

25 Endotoxin is a heat stable bacterial toxin composed of lipopolysaccharides (LPS) released from the outer membrane of Gram-negative bacteria upon lysis (Shenep *et al.*,

- 3 -

J. Infect. Dis., 150(3):380-388, 1984), and is a potent stimulator of the inflammatory response. Endotoxemia occurs when endotoxin enters the bloodstream resulting in a dramatic systemic inflammatory response.

The uptake of oligonucleotides by B lymphocytes has been shown to be regulated by

5 LPS-induced cell activation (Krieg, A.M., *et al.*, *Antisense Res. Devel.* 1:161, 1991). Many detrimental *in vivo* effects of LPS have been shown to result from soluble mediators released by inflammatory cells. (Morrison *et al.*, *Am. J. Pathol.*, 93(2):527-617, 1978). Monocytes and neutrophils, which ingest and kill microorganisms, play a key role in this process. Monocytes and neutrophils respond to
10 endotoxin *in vivo* by releasing soluble proteins with microbicidal, proteolytic, opsonic, pyrogenic, complement-activating and tissue-damaging effects. These factors mediate many of the pathophysiological effects of endotoxin. For example, tumor necrosis factor (TNF), a cytokine released by endotoxin-stimulated monocytes, causes fever, shock, and alterations in glucose metabolism and is a potent stimulator of neutrophils. Other
15 cytokines such as IL-1, IL-6, and IL-8 also mediate many of the pathophysiologic effects of LPS, as well as other pathways involving endothelial cell activation by tissue factor, kininogen, nitric oxide and complement.

Endotoxin-associated disorders result from extra-gastrointestinal exposure to LPS, *e.g.*, administration of LPS-contaminated fluids, inhalation of LPS, or Gram-negative
20 infections. Endotoxin-associated disorders can also result when the natural cellular barrier is injured and the normal Gram-negative flora breach this barrier. For example, endotoxin-associated disorders can occur (a) when there is ischemia of the gastrointestinal tract (*e.g.*, following hemorrhagic shock or during certain surgical procedures), or (b) when systemic or local inflammation causes increased permeability
25 of the gut or lung to endotoxin or Gram-negative organisms. The presence of endotoxin and the resulting inflammatory response may result, for example, in adult respiratory distress syndrome (ARDS), dust-induced airway disease, and exacerbation of asthma, in addition to endotoxemia, systemic inflammatory response syndrome (SIRS), sepsis

syndrome, septic shock, disseminated intravascular coagulation (DIC), cardiac dysfunction, organ failure, liver failure (hepatobiliary dysfunction), brain failure (CNS dysfunction), renal failure, multi-organ failure and shock.

Several therapeutic compounds have been developed to inhibit the toxic effects of 5 endotoxin, including antibacterial LPS-binding agents and anti-LPS antibodies, although each has met with limitations. For example, Polymyxin B (PMB) is a basic polypeptide antibiotic which binds to Lipid A, the most toxic and biologically active component of endotoxin. PMB inhibits endotoxin-mediated activation of neutrophil granule release *in vitro* and is a potential therapeutic agent for Gram-negative infections. However, 10 because of its systemic toxicity, this antibiotic has limited therapeutic use, and is generally used topically. Combination therapy using antibiotics and high doses of methylprednisolone sodium succinate (MPSS) showed more promise as this regimen prevented death in an experimental animal model of Gram-negative sepsis. However, a clinical study using MPSS with antibiotics in treatment of patients having clinical signs 15 of systemic sepsis showed that mortality rates were not significantly different between the treatment and placebo groups (Bone *et al.*, *N. Engl. J. Med.* 317:653, 1987).

SUMMARY OF THE INVENTION

The present invention is based on the finding that nucleic acids containing at least one unmethylated cytosine-guanine (CpG) dinucleotide affect the immune response in a 20 subject by activating natural killer cells (NK) or redirecting a subject's immune response from a Th2 to a Th1 response by inducing monocytic and other cells to produce Th1 cytokines. These nucleic acids containing at least one unmethylated CpG can be used to treat pulmonary disorders having an immunologic component, such as asthma or environmentally induced airway disease.

25 In a first embodiment, a method of treating a subject having or at risk of having an acute decrement in air flow by administering a therapeutically effective amount of nucleic acids containing at least one unmethylated CpG is provided.

- 5 -

In another embodiment, a method of treating a subject having or at risk of having an inflammatory response to lipopolysaccharide by administering a therapeutically effective amount of nucleic acids containing at least one unmethylated CpG is also provided. The invention also provides a method of modifying the level of a cytokine in a subject having 5 or at risk of having inhaled lipopolysaccharide by administering a therapeutically effective nucleic acid containing at least one unmethylated CpG.

In another embodiment, the invention provides a pharmaceutical composition for treating a subject having or at risk of having an inflammatory response to inhaled lipopolysaccharide including a nucleic acid sequence containing at least one 10 unmethylated CpG in a pharmacologically acceptable carrier.

In a further embodiment, isolated nucleic acid sequences as set forth in SEQ ID NOS:2, 17, 18, 59-65 are provided.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow chart showing the pathways to sepsis and acute lung injury.

15 FIG. 2 is a graph plotting the concentration of cytokines (TNF- α , MIP-2, IL-10, IL-12, and IFN- γ) in the serum four hours after intravenous treatment with either an oligonucleotide containing embedded CpG motifs or an oligonucleotide without CpG motifs. Serum samples were obtained immediately following an inhalation challenge with *E. coli* LPS. Error bars show Standard Error (SE).

20 FIG. 3 is a graph plotting the concentration of total cells and PMNs in the whole lung lavage fluid following inhalation challenge with *Escherichia coli* LPS. Thirty minutes, four hours and 12 hours prior to the inhalation challenge, mice were either treated with an oligonucleotide containing embedded CpG motifs or were treated with an oligonucleotide without CpG motifs. Error bars show SE.

- 6 -

FIG. 4 is a graph showing the concentration of cytokines (TNF- α , MIP-2, and IL-12) in the whole lung lavage fluid following inhalation of *E. coli* LPS. Mice were pretreated with an oligonucleotide containing embedded CpG motifs or were pretreated with an oligonucleotide without CpG motifs four hours prior to inhalation challenge with LPS.

5 Error bars show SE.

FIG. 5 is an autoradiograph showing an RNase protection assay of the total mRNA isolated from lungs of mice exposed to *E. coli* LPS by inhalation. Mice were pretreated with an oligonucleotide containing embedded CpG motifs or were pretreated with an oligonucleotide without CpG motifs four hours prior to inhalation challenge with LPS.

10 L32 encodes a ribosomal protein and was used to assess the concentration of RNA loaded onto the gel.

FIG. 6 is a graph plotting the concentration of total cells and PMNs in the whole lung lavage fluid following inhalation of *E. coli* LPS. Mice were pretreated with an oligonucleotide containing embedded CpG motifs or were pretreated with an oligonucleotide without CpG motifs four hours prior to inhalation challenge with LPS.

15 Error bars show SE.

FIG. 7 is a graph showing the concentration of total cells and PMNs in the whole lung lavage fluid following inhalation of *E. coli* LPS. C57BL/6 mice and IL-10 knockout mice (C57BL/6-IL10^{tm1Cgn}) were pretreated with either an oligonucleotide containing 20 embedded CpG motifs or with intravenous saline four hours prior to inhalation challenge with LPS. Error bars show SE.

FIG. 8 is a graph plotting lung lavage cell count over time. The graph shows that when the mice are initially injected intraperitoneally (i.p.) with *Schistosoma mansoni* eggs "egg," which induces a Th2 immune response, and subsequently inhale *Schistosoma mansoni* egg antigen "SEA" (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given CpG ODN along with egg, the

- 7 -

inflammatory cells in the lung are not as increased by subsequent inhalation of SEA (open triangles).

FIG. 9 is a graph plotting lung lavage eosinophil count over time. Again, the graph shows that when the mice are initially injected with egg and subsequently inhale SEA (open circle), many eosinophils are present in the lungs. However, when the mice are initially given CpG ODN along with egg, the inflammatory cells in the lung are not as increased by subsequent inhalation of the SEA (open triangles).

FIG. 10 is a bar graph plotting the effect on the percentage of macrophage, lymphocyte, neutrophil and eosinophil cells induced by exposure to saline alone; egg, then SEA; egg and CpG ODN, then SEA; and egg and control oligo, then SEA. When the mice are treated with the control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 11 is a bar graph plotting eosinophil count in response to injection of various amounts of the protective oligo SEQ ID NO: 10.

FIG. 12 is a graph plotting interleukin 4 (IL-4) production (pg/ml) in mice over time in response to injection of egg, then SEA (open diamond); egg and CpG ODN, then SEA (open circle); or saline, then saline (open square). The graph shows that the resultant inflammatory response correlates with the levels of the Th2 cytokine IL-4 in the lung.

FIG. 13 is a bar graph plotting interleukin 12 (IL-12) production (pg/ml) in mice over time in response to injection of saline; egg, then SEA; or CpG ODN and egg, then SEA. The graph shows that administration of an oligonucleotide containing an unmethylated

CpG motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

FIG. 14 is a bar graph plotting interferon gamma (IFN- γ production (pg/ml) in mice over time in response to injection of saline; egg, then saline; or CpG ODN and egg, then SEA.

5 The graph shows that administration of an oligonucleotide containing an unmethylated CpG motif can also redirect the cytokine response of the lung to production of IFN- γ , indicating a Th1 type of immune response.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It is to be understood that this invention is not limited to the particular methodology, 10 protocols, sequences, models and reagents described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

All publications mentioned herein are incorporated herein by reference for the purpose 15 of describing and disclosing the oligonucleotides and methodologies which are described in the publications which might be used in connection with the presently described invention.

The binding of DNA to cells has been shown to be similar to a ligand receptor interaction: binding is saturable, competitive, and leads to DNA endocytosis and 20 degradation into oligonucleotides (Bennet, R.M., *et al.*, *J. Clin. Invest.* 76:2182, 1985). Like DNA, oligodeoxyribonucleotides are able to enter cells in a process which is sequence, temperature, and energy independent (Jaroszewski and Cohen, *Ad. Drug Del. Rev.* 6:235, 1991). An "oligodeoxyribonucleotide" as used herein is a deoxyribonucleic acid sequence from about 3-50 bases in length. Lymphocyte oligodeoxyribonucleotide 25 uptake has been shown to be regulated by cell activation (Krieg, A.M., *et al.*, *Antisense*

Research and Development 1:161, 1991). The present invention is based on the finding that certain oligonucleotides (ODN) containing at least one unmethylated cytosine-guanine (CpG) dinucleotide activate the immune response.

In one embodiment, the invention provides a method for treating a subject having or at 5 risk of having an acute decrement in air flow by administering a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG. The term "nucleic acid" or "oligonucleotide" refers to a polymeric form of nucleotides at least five bases in length. The nucleotides of the invention can be deoxyribonucleotides, ribonucleotides, or modified forms of either nucleotide. Generally, double-stranded 10 molecules are more stable *in vivo*, while single-stranded molecules have increased activity.

The nucleic acid molecule can include the use of phosphorothioate or phosphorodithioate rather than phosphodiester linkages within the backbone of the molecule, or methylphosphorothioate terminal linkages (Krieg, A.M., *et al.*, *Antisense and Nucl Acid 15 Drug Dev* 6:133-9, 1996; Boggs, R.T., *et al.*, *Antisense and Nucl Acid Drug Dev*, 7:461-71, 1997). The phosphate backbone modification can occur at the 5' end of the nucleic acid, for example at the first two nucleotides of the 5' end of the nucleic acid. The phosphate backbone modification may occur at the 3' end of the nucleic acid, for example at the last five nucleotides of the 3' end of the nucleic acid. International Patent 20 Application WO 95/26204, entitled "Immune stimulation by phosphorothioate oligonucleotide analogs" reports the nonsequence-specific immunostimulatory effect of phosphorothioate modified oligonucleotides. Nontraditional bases such as inosine and queosine, as well as acetyl-, thio- and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine can also be included, which are not as easily recognized 25 by endogenous endonucleases. Other stabilized nucleic acid molecules include: nonionic DNA analogs, such as alkyl- and aryl-phosphonates (in which the charged oxygen moiety is alkylated). Nucleic acid molecules which contain a diol, such as tetrahyleneglycol or

- 10 -

hexaethyleneglycol, at either or both termini are also included. The term "oligonucleotide" includes both single and double-stranded forms of DNA.

A "CpG" or "CpG motif" refers to a nucleic acid having a cytosine followed by a guanine linked by a phosphate bond. The term "methylated CpG" refers to the 5 methylation of the cytosine on the pyrimidine ring, usually occurring the 5-position of the pyrimidine ring. The term "unmethylated CpG" refers to the absence of methylation of the cytosine on the pyrimidine ring. Methylation, partial removal, or removal of an unmethylated CpG motif in an oligonucleotide of the invention is believed to reduce its effect. Methylation or removal of all unmethylated CpG motifs in an oligonucleotide 10 substantially reduces its effect. The effect of methylation or removal of a CpG motif is "substantial" if the effect is similar to that of an oligonucleotide that does not contain a CpG motif.

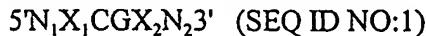
Preferably the CpG oligonucleotide is in the range of about 8 to 30 bases in size. For use in the instant invention, the nucleic acids can be synthesized *de novo* using any of a 15 number of procedures well known in the art. For example, the *b*-cyanoethyl phosphoramidite method (Beaucage, S.L., and Caruthers, M.H., *Tet. Let.* 22:1859, 1981); nucleoside H-phosphonate method (Garegg *et al.*, *Tet. Let.* 27:4051-4054, 1986; Froehler *et al.*, *Nucl. Acid. Res.* 14:5399-5407, 1986; Garegg *et al.*, *Tet. Let.* 27:4055-4058, 1986, Gaffney *et al.*, *Tet. Let.* 29:2619-2622, 1988). These chemistries can be performed by 20 a variety of automated oligonucleotide synthesizers available in the market. Alternatively, CpG dinucleotides can be produced on a large scale in plasmids, (see Sambrook, T., *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor laboratory Press, New York, 1989) which after being administered to a subject are degraded into oligonucleotides. Oligonucleotides can be prepared from existing nucleic 25 acid sequences (e.g., genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

- 11 -

For use *in vivo*, nucleic acids are preferably relatively resistant to degradation (e.g., via endo-and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized nucleic acid has at least 5 a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries. Aryl-and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) 10 can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A., *Chem. Rev.* 90:544, 1990; Goodchild, J., *Bioconjugate Chem.* 1:165, 1990).

For administration *in vivo*, nucleic acids may be associated with a molecule that results 15 in higher affinity binding to target cell (e.g., B-cell, monocytic cell and natural killer (NK) cell) surfaces and/or increased cellular uptake by target cells to form a "nucleic acid delivery complex." Nucleic acids can be ionically or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or cross-linking agents can be used, e.g., protein A, carbodiimide, and 20 N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP). Nucleic acids can alternatively be encapsulated in liposomes or virosomes using well-known techniques.

In one embodiment, the nucleic acid sequences useful in the methods of the invention are represented by the formula:



25 wherein at least one nucleotide separates consecutive CpGs; X₁ is adenine, guanine, or thymidine; X₂ is cytosine or thymine, N is any nucleotide and N₁ + N₂ is from about 0-26 bases. In a preferred embodiment, N₁ and N₂ do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in

- 12 -

length. However, nucleic acids of any size (even may kb long) can be used in the invention if CpGs are present, as larger nucleic acids are degraded into oligonucleotides inside cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' or 3' terminals and/or the consensus 5 mitogenic CpG motif is not a palindrome. A "palindromic sequence" or "palindrome" means an inverted repeat (*i.e.*, a sequence such as ABCDEE'D'C'B'A', in which A and A' are bases capable of forming the usual Watson-Crick base pairs. An exemplary nucleic acid sequence of the invention is:

5'-ATAATCGACGTTCAAGCAAG-3' (SEQ ID NO:2).

10 In another embodiment, the method of the invention includes the use of an oligonucleotide which contains a CpG motif represented by the formula:

5' N₁X₁X₂CGX₃X₄N₂ 3' (SEQ ID NO:3)

wherein at least one nucleotide separates consecutive CpGs; X₁X₂ is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X₃X₄ is selected from the group 15 consisting of TpT or CpT; N is any nucleotide and N₁ + N₂ is from about 0-26 bases. In a preferred embodiment, N₁ and N₂ do not contain a CCGG quadmer or more than one CCG or CGG trimer. CpG ODN are also preferably in the range of 8 to 30 bases in length, but may be of any size (even many kb long) if sufficient motifs are present, since such larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred 20 synthetic oligonucleotides of this formula do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5' and/or 3' terminals and/or the consensus mitogenic CpG motif is not a palindrome. Other CpG oligonucleotides can be assayed for efficacy using methods described herein.

A prolonged effect can be obtained using stabilized oligonucleotides, where the 25 oligonucleotide incorporates a phosphate backbone modification (*e.g.*, a phosphorothioate or phosphorodithioate modification). More particularly, the phosphate backbone modification occurs at the 5' end of the nucleic acid for example, at the first two nucleotides of the 5' end of the nucleic acid. Further, the phosphate backbone

- 13 -

modification may occur at the 3' end of the nucleic acid for example, at the last five nucleotides of the 3' end of the nucleic acid. Preferred nucleic acids containing an unmethylated CpG have a relatively high stimulation with regard to B cell, monocyte, and/or natural killer cell responses (e.g., induction of cytokines, proliferative responses, 5 lytic responses, among others).

The "stimulation index" is a measure of a CpG ODN to effect an immune response which can be tested in various immune cell assays. The stimulation of the immune response can be assayed by measuring various immune parameters, e.g., measuring the antibody-forming capacity, number of lymphocyte subpopulations, mixed leukocyte response 10 assay, lymphocyte proliferation assay. The stimulation of the immune response can also be measured in an assay to determine resistance to infection or tumor growth. Methods for measuring a stimulation index are well known to one of skill in the art. For example, one assay is the incorporation of ^3H uridine in a murine B cell culture, which has been contacted with a 20 μM of oligonucleotide for 20h at 37°C and has been pulsed with 1 μCi 15 of ^3H uridine; and harvested and counted 4h later. The induction of secretion of a particular cytokine can also be used to assess the stimulation index. Without meaning to be bound by theory, for use *in vivo*, for example to treat a subject having or at risk of having an acute decrement in air flow in response to endotoxin, it is important that the CpG ODN be capable of effectively inducing cytokine secretion by monocytic cells 20 and/or Natural Killer (NK) cell lytic activity. In one method, the stimulation index of the CpG ODN with regard to B-cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20, while recognizing that there are differences in the stimulation index among individuals.

The CpG ODN of the invention stimulate cytokine production (e.g., IL-6, IL-12, IFN- γ , 25 TNF- α and GM-CSF). Exemplary sequences include:

TCCATGTCGCTCCTGATGCT (SEQ ID NO:4),
TCCATGTCGTTCTGATGCT (SEQ ID NO:5), and
TCGTCGTTTGTGCGTTTGTGCTT (SEQ ID NO:6).

- 14 -

The CpG ODN of the invention are also useful for stimulating natural killer-cell (NK) lytic activity in a subject such as a human. Specific, but nonlimiting examples of such sequences include:

5 TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:7),
TCGTCGTTTGTCGTTTGTCGTT (SEQ ID NO:6),
TCGTCGTTGTCGTTTGTCGTT (SEQ ID NO:8),
GCGTGCCTGTCGTTGTCGTT (SEQ ID NO:9),
TGTTCGTTGTCGTTGTCGTT (SEQ ID NO:10),
TGTTCGTTGTCGTTGTCGTT (SEQ ID NO:11), and
10 TCGTCGTCGTCGTT (SEQ ID NO:12).

The nucleic acid sequences of the invention are also useful for stimulating B cell proliferation. Specific, but nonlimiting examples of such sequences include:

15 TCCTGTCGTTCCCTTGTCGTT (SEQ ID NO:13),
TCCTGTCGTTTTTGTCGTT (SEQ ID NO:14),
TCGTCGCTGTCGCCCTCTT (SEQ ID NO:15),
TCGTCGCTGTTGTCGTTCTT (SEQ ID NO:16),
TCGTCGTTTGTCGTTTGTCGTT (SEQ ID NO:6),
TGTTCGTTGTCGTTGTCGTT (SEQ ID NO:8) and
TGTTCGTTGTCGTTGTCGTT (SEQ ID NO:11).

20 Preferred CpG ODN can effect at least about 500 pg/ml of TNF- α , 15 pg/ml IFN- γ , 70 pg/ml of GM-CSF 275 pg/ml of IL-6, 200 pg/ml IL-12, depending on the therapeutic indication. These cytokines can be measured by assays well known in the art. The ODNs listed above or other preferred CpG ODN can effect at least about 10%, more preferably at least about 15% and most preferably at least about 20% YAC-1 cell specific lysis or
25 at least about 30%, more preferably at least about 35%, and most preferably at least about 40% 2C11 cell specific lysis, in assays well known in the art (see Example 4).

The term "acute" refers to a condition having a short and relatively severe course. A "decrement in air flow" is a decrease in a measurable parameter of lung function. The

- 15 -

terms "lung function" and "pulmonary function" are used interchangeably and shall be interpreted to mean physically measurable operations of a lung including but not limited to inspiratory flow rate, expiratory flow rate, and lung volume. Methods of quantitatively determining pulmonary function are used to measure lung function.

5 Methods of measuring pulmonary function most commonly employed in clinical practice involve timed measurement of inspiratory and expiratory maneuvers to measure specific parameters. For example, forced vital capacity (FVC) measures the total volume in liters exhaled by a patient forcefully from a deep initial inspiration. This parameter, when evaluated in conjunction with the forced expired volume in one second (FEV₁), allows

10 bronchoconstriction to be quantitatively evaluated. A problem with forced vital capacity determination is that the forced vital capacity maneuver (*i.e.*, forced exhalation from maximum inspiration to maximum expiration) is largely technique dependent. In other words, a given patient may produce different FVC values during a sequence of consecutive FVC maneuvers. The FEF 25-75 or forced expiratory flow determined over

15 the midportion of a forced exhalation maneuver tends to be less technique dependent than the FVC. Similarly, the FEV₁ tends to be less technique dependent than FVC. In addition to measuring volumes of exhaled air as indices of pulmonary function, the flow in liters per minute measured over differing portions of the expiratory cycle can be useful in determining the status of a patient's pulmonary function. In particular, the peak

20 expiratory flow, taken as the highest air flow rate in liters per minute during a forced maximal exhalation, is well correlated with overall pulmonary function in a patient with asthma and other respiratory diseases.

The term "asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

By "therapeutically effective amount" is meant the quantity of a compound according to the invention necessary to prevent, to cure or at least partially arrest symptoms in a

- 16 -

subject. A subject is any mammal, preferably a human. Amounts effective for therapeutic use will, of course, depend on the severity of the disease and the weight and general state of the subject. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Gilman *et al.*, eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference.

- 10 10 An oligonucleotide containing at least one unmethylated CpG can be used alone to activate the immune response or can be administered in combination with another therapeutic modality, either a drug or a surgical procedure. For example, when the oligonucleotide containing at least one unmethylated CpG is administered in conjunction with another therapeutic modality, the oligonucleotide can be administered before, after, and/or simultaneously with the other therapeutic modality. The oligonucleotide containing at least one unmethylated CpG can have an additional efficacy (*e.g.*, through antisense or other means) in addition to its ability to activate the immune response.

In another embodiment, the invention further provides a method of treating a subject having or at risk of having an inflammatory response to LPS by administering to the subject a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG.

Examples of diseases which can be associated with Gram-negative bacterial infections or endotoxemia include bacterial meningitis, neonatal sepsis, cystic fibrosis, inflammatory bowel disease and liver cirrhosis, Gram-negative pneumonia, 25 Gram-negative abdominal abscess, hemorrhagic shock and disseminated intravascular coagulation. Subjects who are leukopenic or neutropenic, including subjects treated with chemotherapy or immunocompromised subjects (for example with AIDS), are

particularly susceptible to bacterial infection and the subsequent effects of endotoxin.

By "lipopolysaccharide" or "LPS" is meant a compound composed of a heteropolysaccharide (which contains somatic O antigen) covalently bound to a phospholipid moiety (lipid a). LPS is a major component of the cell wall of 5 Gram-negative bacteria. By "endotoxin" is meant a heat-stable toxin associated with the outer membranes of certain Gram-negative bacteria, including the enterobacteria, brucellae, neisseriae, and vibrios. Endotoxin, normally released upon disruption of the bacterial cells, is composed of lipopolysaccharide molecules (LPS) and any associated proteins. The phospholipid moiety of LPS, lipid a, is associated with LPS toxicity.

10 When injected in large quantities endotoxin produces hemorrhagic shock and severe diarrhea; smaller amounts cause fever, altered resistance to bacterial infection, leukopenia followed by leukocytosis, and numerous other biologic effects. Endotoxin is a type of "bacterial pyrogen," which is any fever-raising bacterial product. The terms "endotoxin," "LPS," and "lipopolysaccharide" as used herein are essentially synonymous.

15 The invention further provides a method of treating a subject having or at risk of having an inflammatory response to LPS. It is known that LPS produces an inflammatory response in normal and asthmatic patients. By "inflammatory response" is meant an accumulation of white blood cells, either systemically or locally at the site of inflammation. The inflammatory response may be measured by many methods well 20 known in the art, such as the number of white blood cells (WBC), the number of polymorphonuclear neutrophils (PMN), a measure of the degree of PMN activation, such as luminal enhanced-chemiluminescence, or a measure of the amount of cytokines present. The term "cytokine" is used as a generic name for a diverse group of soluble 25 proteins and peptides which act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Examples of cytokines include, but are not limited to, TNF- α , IL-10,

- 18 -

IL-12, interferon- γ . Importantly, interferon- γ is a key cytokine mediating LPS-induced inflammation (e.g., Ozmen, L., *et al.*, *J. Exp. Med.* 180:907-915, 1994). The release of interferon- γ is induced by IL-12 derived from macrophage/monocyte/dendritic cells. (e.g., Balanchard, D.K., *et al.*, *J. Immunol.* 136:963-970, 1986), and IL-10 inhibits 5 interferon- γ via a macrophage-dependent step in which IL-12 production is inhibited (D'Andrea, a., *et al.*, *J. Exp. Med.* 178:1041-1048, 1993). Without wanting to be bound by theory, it is possible that nucleic acids containing unmethylated CpG could reduce the inflammatory response to LPS by increasing the production and response of IL-10, or by modulating the response of a factor which in turn increase the production and response 10 of IL-10 or IL-6.

The invention further provides a method of modulating the level of a cytokine altered in response to inhaled LPS. The term "modulate" envisions the suppression of expression of a particular cytokine when it is overexpressed, or augmentation of the expression of a particular cytokine when it is underexpressed. Modulation of a particular cytokine can 15 occur locally or systemically. It is believed that the CpG oligonucleotides do not directly activate purified NK cells, but rather render them competent to respond to IL-12 with a marked increase in their IFN- γ production. By inducing IL-12 production and the subsequent increased IFN- γ secretion by NK cells, the immunostimulatory nucleic acids also promote a Th1 type immune response. No direct activation of proliferation or 20 cytokine secretion by highly purified T cells has been found. Cytokine profiles determine T cell regulatory and effector functions in immune responses.

- 19 -

Cytokines also play a role in directing the T cell response. Helper (CD4⁺) T cells orchestrate the immune response of mammals through production of soluble factors that act on other immunē system cells, including other T cells. Most mature CD4⁺ T helper cells express one of two cytokine profiles: Th1 or Th2. Th1 cells secrète IL-2, IL-3, 5 IFN- γ , TNF- β , GM-CSF and high levels of TNF- α . Th2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, GM-CSF and low levels of TNF- α . The Th1 subset promotes delayed-type hypersensitivity, cell-mediated immunity, and immunoglobulin class switching to IgG_{2a}. The Th2 subset induces humoral immunity by activating B cells, promoting antibody production, and inducing class switching to IgG₁ and IgE.

10 Several factors have been shown to influence commitment to Th1 or Th2 profiles. The best characterized regulators are cytokines. IL-12 and IFN- γ are positive Th1 and negative Th2 regulators. IL-12 promotes IFN- γ production, and IFN- γ provides positive feedback for IL-12. IL-4 and IL-10 appear to be required for the establishment of the Th2 cytokine profile and to down-regulate Th1 cytokine production; the effects of IL-4 15 are in some cases dominant over those of IL-12. IL-13 was shown to inhibit expression of inflammatory cytokines, including IL-12 and TNF- α by LPS-induced monocytes, in a way similar to IL-4. The IL-12 p40 homodimer binds to the IL-12 receptor and antagonizes IL-12 biological activity; thus it blocks the pro-Th1 effects of IL-12.

20 The invention may be used to treat individuals who are "at risk" of developing a acute decrement in airflow or who are at risk of LPS exposure. These individuals may be identified by any diagnostic means, or by epidemiological evidence such as exposure data. These individuals may be treated by a method of the invention prior to, at the time of, or after the actual onset of the clinical appearance. The "clinical appearance" can be any sign or symptom of the disorder.

25 This invention further provides administering to a subject having or at risk of having an inflammatory response to inhaled LPS, a therapeutically effective dose of a pharmaceutical composition containing the compounds of the present invention and a

- 20 -

pharmaceutically acceptable carrier. "Administering" the pharmaceutical composition of the present invention may be accomplished by any means known to the skilled artisan.

The pharmaceutical compositions according to the invention are in general administered topically, intravenously, orally, parenterally or as implants, and even rectal use is

5 possible in principle. Suitable solid or liquid pharmaceutical preparation forms are, for example, granules, powders, tablets, coated tablets, (micro)capsules, suppositories, syrups, emulsions, suspensions, creams, aerosols, drops or injectable solution in ampule form and also preparations with protracted release of active compounds, in whose preparation excipients and additives and/or auxiliaries such as disintegrants, binders,

10 coating agents, swelling agents, lubricants, flavorings, sweeteners or solubilizers are customarily used as described above. The pharmaceutical compositions are suitable for use in a variety of drug delivery systems. For a brief review of present methods for drug delivery, see Langer, *Science* 249:1527-1533, 1990, which is incorporated herein by reference.

15 The pharmaceutical compositions are preferably prepared and administered in dose units. Solid dose units are tablets, capsules and suppositories. For treatment of a patient, depending on activity of the compound, manner of administration, nature and severity of the disorder, age and body weight of the patient, different daily doses are necessary. Under certain circumstances, however, higher or lower daily doses may be appropriate.

20 The administration of the daily dose can be carried out both by single administration in the form of an individual dose unit or else several smaller dose units and also by multiple administration of subdivided doses at specific intervals.

The pharmaceutical compositions according to the invention may be administered locally or systemically. By "therapeutically effective dose" is meant the quantity of a compound

25 according to the invention necessary to prevent, to cure or at least partially arrest the symptoms of the disorder and its complications. Amounts effective for this use will, of course, depend on the severity of the disease and the weight and general state of the

- 21 -

patient. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in situ* administration of the pharmaceutical composition, and animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, *e.g.*, in Gilman *et al.*, eds., Goodman And Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990, each of which is herein incorporated by reference.

The following examples are intended to illustrate but not to limit the invention in any manner, shape, or form, either explicitly or implicitly. While they are typical of those 10 that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1

METHODS FOR PULMONARY RESPONSE TO INHALED LPS

In the first series of experiments, mice were intravenously treated with 20 base pair (bp) 15 oligonucleotides containing CpG motifs (CpG oligo) or 20 bp oligonucleotides without embedded CpG motifs (non CpG oligo) 30 min, 4 hours, or 12 hours prior to a 4 hour inhalation challenge with *E. coli* LPS (1.5 μ g/m³). To determine whether unmethylated CpG motifs were responsible for the protective effect, we pretreated mice with oligonucleotides containing either unmethylated CpG motifs or methylated CpG motifs 20 prior to an inhalation challenge with *E. coli* LPS. Finally, to determine the role of IL-10, we pretreated IL-10 knockout mice with CpG oligos and then performed a similar inhalation challenge with *E. coli* LPS. Immediately post inhalation challenge, all mice were sacrificed, blood samples were obtained, whole lung lavage was performed, and lungs were harvested for mRNA analysis.

25 Animals. C3H/HeBFEJ, C57BL/6, and C57BL/6-IL10^{tm1Cgn} male mice (Jackson Laboratories, Bar Harbor, ME) were obtained at 6 weeks of age and used within 2 weeks. All animal care and housing requirements set forth by the National Institutes of Health

- 22 -

Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Mice were maintained in wood-chip bedding (Northeastern Product, Warrensberg, NY), with food (Formulab Chow 5008, 5 PMI, Richmond, IN) and water supplied ad libitum.

Oligonucleotides. Twenty base pair oligonucleotides were synthesized with and without the embedded CpG motifs (Oligos etc., Wilsonville, OR). These oligonucleotides contained a nuclease-resistant phosphorothioate-modified backbone, and were purified by two rounds of ethanol precipitation prior to use. The CpG dinucleotide was flanked 10 by two 5' purines and two 3' pyrimidines to enhance the stimulatory effect of the oligonucleotide. The "nonstimulatory" oligonucleotide was identical to the stimulatory oligonucleotide except that the two embedded CpG motifs were modified, one appearing as an ApG motif and the other appearing as a GpC motif. The two synthesized oligonucleotides had the following sequences:

15 CpG Oligonucleotide: ATAATCGACGTTCAAGCAAG (SEQ ID
NO:17)
Non-CpG oligonucleotide: ATAATAGAGCTTCAAGCAAG (SEQ ID
NO:18)

Methylation Protocol. DNA was methylated as we have described previously (Krieg, 20 A.M., *et al.*, *Nature* 374:546-9, 1995) with 2 U CpG methylase (New England Biolabs; Beverly, MA) per μ g DNA for 18 hours at 37°C. Methylated DNA was tested to confirm that it was completely protected against digestion with Hpa-II but not Msp-I.

Chemicals. Endotoxin was purchased as lyophilized purified *E. coli* 0111:B4 25 lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, MO, PN# L2630) and had a specified activity of 1.3×10^6 ng/mg and protein content less than 3%.

- 23 -

Endotoxin Assay. The endotoxin concentrations of LPS solution, LPS aerosol, and oligonucleotides were assayed using the chromogenic *Limulus* amebocyte lysate (LAL) assay (QCL-1000, Whittaker Bioproducts, Inc., Walkersville, MD) with sterile, pyrogen-free labware and a temperature controlled microplate block and microplate reader (405 nm). The LPS solution was serially diluted in pyrogen-free water and assayed. The airborne concentration of LPS was assessed by sampling 0.30 m³ of air drawn from the exposure chamber through 47 mm binder-free glass microfiber filters (EPM-2000, Whatman Intl. Ltd., Maidstone, England) held within a 47 mm stainless steel in-line air sampling filter holder (Gelman Sciences Inc., Ann Arbor, MI). Air sampling filters were extracted with 10 ml of pfw at room temperature with gentle shaking for 1 hour. They were then serially diluted with pfw and assayed for endotoxin. Four to 6 air samples were assayed for each exposure. All standard curves (0.1 to 1.0 EU/ml) achieved a linear regression coefficient exceeding $r=0.995$. Spiked samples and filter blanks and participates were run routinely; interlaboratory validation studies were also performed routinely.

Exposure Protocol and Monitoring Equipment. LPS aerosols were generated into a glass 20 L exposure chamber using a PITT#1 nebulizer supplied with extract by a syringe pump. Liquid feed rates ranged from 0.0027 to 0.21 ml/min. HEPA-filtered air was supplied to the nebulizer at flow rates ranging from 10 to 17 L/min. Mixing within the chamber was aided by a magnetically coupled rotor. The chamber atmosphere was exchanged at 1 change/min. LPS concentrations were determined by sampling the total chamber outflow. Particle size distributions were determined with an Aerodynamic Particle Sizer (TSI, Inc., St. Paul, MN) and gravimetrically with a Marble personal cascade impactor and Mylar media (Thorne, P.S., *Am. J. Ind. Med.* 25:109-112, 1997) by sampling within the exposure chamber.

Lung Lavage. Immediately following the inhalation challenge, mice were euthanized, the chest was opened, and lungs were lavaged in situ via PE-90 tubing inserted into the exposed trachea. A pressure of 25 cm H₂O was used to wash the lungs with 6.0 ml of

- 24 -

sterile pyrogen free saline. Following whole lung lavage, the lungs were isolated and frozen in liquid nitrogen and stored at -70°C.

Treatment of Bronchoalveolar Lavage Fluid. A standard method (Schwartz, D.A., *et al.*, *Am. J. Physiol.* 267:L609-617, 1994) of processing the sample was as follows:

5 immediately following lavage, the volume was noted and 15 ml conical tubes were centrifuged for 5 min at 200 x g. The supernatant fluid was decanted and frozen at -70°C for subsequent use. The residual pellet of cells was resuspended and washed twice in HBSS (without Ca⁺⁺ or Mg⁺⁺). After the second wash, a small aliquot of the sample was taken for cell count using a hemocytometer. The cells were then washed once more and

10 resuspended in RPMI medium so that the final concentration gave a cell count of 1×10^6 cells/ml. The cells which were present in 10-12 μ l of the 1×10^6 ml cell suspension were spun for 5 minutes onto a glass slide using a special filter card using a cytocentrifuge (Cytospin-2; Shandon Southern, Sewickley, PA). Staining was carried out using a Diff Quick Stain Set (Harleco, Gibbstown, NY). The slide was then dried, one drop of

15 optically clear immersion oil was put on the slide over the cells and a coverslip was placed on top.

Cytokine Analysis of lavage fluid and serum. Lavage fluid was assayed for TNF- α , MIP-2, IL-6, IL-10, IL-12, and IFN- γ . In all cases, a polyclonal antibody specific for the murine recombinant cytokine (TNF- α , MIP-2, IL-6, IL-10, IL-12, or IFN- γ) was used

20 as a capture reagent in a standard commercially available sandwich ELISA (R & D Systems; Minneapolis, MN). The limit for detection for TNF- α is 5.1 pg/ml, MIP-2 is 1.5 pg/ml, IL-6 is 10 pg/ml, IL-10 is 10 pg/ml, IL-12 is 5 pg/ml, and IFN- γ is 10 pg/ml.

Preparation of RNA and Multiprobe RNase Protection Assay. Total RNA was extracted from lung specimens using the single-step method (Chomczynski and Pandsacchi, *Anal Biochem* 162:156-9, 1987; Kedzierski, W., *Biotechniques* 10:210-214, 1991), lysing flash frozen lung in RNA STAT-60 (Tel-Test B; Friendswood, TX). The composition of RNA STAT-60 includes phenol and guanidinium thiocyanate in a monophase solution. The

- 25 -

lung parenchyma was homogenized in the RNA STAT-60 using a polytron homogenizer. Chloroform was added, the total RNA was precipitated from the aqueous phase by addition of isopropanol, and the total RNA was washed with ethanol and solubilized in water. After drying the pellet in a vacuum desiccator, the yield and purity of RNA was 5 quantitated by measuring the ratio of absorbances at 260 and 280 nm. Mini-gel electrophoresis was used to confirm the integrity of the 28s and 18s rRNA bands. Gene transcripts were detected using the RNase protection assay as previously described (Hobbs, J.M., et al., *J. Immunol.* 150:3602, 1993). Equivalent amounts of RNA were examined, as judged by the amount of L32, which encodes an ubiquitously expressed 10 ribosome subunit protein (Rajchel, A., et al., *Nucl. Acid. Res.* 16:2347, 1987) in each sample. Commercially available probes were used to detect TNF- α , MIP-2, IL-6, IL-10, IL-12, and IFN- γ .

Statistical Analysis. Three comparisons were pursued in this analysis: 1) the effect of intravenous CpG containing oligonucleotides versus oligonucleotides without embedded 15 CpG motifs in modulating the inflammatory response to inhaled LPS; 2) the effect of unmethylated CpG motifs versus methylated CpG motifs in controlling the inflammatory response to LPS; and 3) the role of IL-10 in mediating the protective effect of unmethylated CpG containing oligonucleotides. The inflammatory response was assessed using lavage cellularity, lavage fluid cytokine concentration, serum 20 concentration of cytokines, and the relative concentration of mRNA for specific cytokines in the lung parenchyma. After making sure that the data were normally distributed, statistical comparisons were made using parametric statistics including the Student's T-test (Rosner, R., Fundamentals of Biostatistics (3rd edition) Boston, MA, PWS-Kent, 1980).

- 26 -

EXAMPLE 2

CpG ODN REDUCES THE PULMONARY RESPONSE TO INHALED
LPS AND STIMULATES THE IMMUNE RESPONSE

Pretreatment with CpG oligonucleotides (ODN) resulted in a systemic inflammatory response. Although intravenous treatment with CpG ODN did not affect the concentration of peripheral white blood cells; compared to non-CpG ODN, treatment with CpG ODN prior to LPS inhalation resulted in a higher concentration of PMNs 30 min, 4 hours, and 12 hours after injection. As expected, intravenous treatment with CpG oligonucleotides also affected the concentration of cytokines in the serum.

10 Compared to non-CpG ODN, CpG ODN resulted in an increase in the concentration of MIP-2, IL-10, and IL-12 in the serum of mice following LPS inhalation (FIG. 1). These differences were most pronounced 30 min and 4 hours after intravenous administration but were still present 12 hours after exposure to CpG containing oligonucleotides. No differences were observed for the serum concentration of TNF- α , IL-6, and IFN- γ at any

15 of the time points in mice pre-treated with either oligonucleotide and then exposed to LPS (data not shown for IL-6).

Pretreatment with CpG containing oligonucleotides reduced the pulmonary response to inhaled LPS. Animals pretreated with CpG oligonucleotides at 0.5, 4, and 12 hours had a reduced concentration of cells in the lavage fluid following inhalation challenge with

20 LPS (FIG. 2). However, this effect appeared to be time dependent since pretreatment with oligonucleotide at 0.5 and 4 hours resulted in a reduced percentage of lavage PMNs while pretreatment with the oligonucleotide 12 hours prior to the inhalation challenge did not affect the percentage of lavage PMNs (FIG. 2). Although pretreatment with CpG containing oligonucleotides resulted in significant changes in the concentration of

25 cytokines in the lavage fluid, the changes in cytokine concentration were predominantly evident when mice were pretreated with CpG oligonucleotides 4 hours prior to the inhalation challenge. While significant reductions were observed in the concentration of TNF- α and MIP-2, the lavage fluid concentration of IL-12 was elevated following

- 27 -

treatment with CpG oligonucleotides 4 hours prior to the inhalation challenge (FIG. 3). IL-6, IL-10, and IFN- γ were not measurable in the lavage fluid following inhalation of LPS at any of the time points. Interestingly, results from the RNase protection assay indicate that total lung mRNA concentrations for TNF- α , MIP-2, IL-6, IL-10, and IFN- γ 5 are similar in mice pretreated with the CpG and non-CpG containing oligonucleotide (FIG. 4). These results also demonstrate that mRNA IL-12 appears to be upregulated in the lung only from mice pretreated with CpG containing oligonucleotides.

To determine the specificity of the CpG oligonucleotides in suppressing the inflammatory response to inhaled LPS, the CpG motifs were methylated. The 10 immunosuppressive effects of two identical oligonucleotides, one with unmethylated CpG motifs and the other with methylated CpG motifs, were compared. Methylating the CpG motifs abolished the protective effect of CpG oligonucleotides in preventing the cellular inflammatory response to inhaled LPS (FIG. 5).

Further experiments were conducted with the following oligonucleotides:

15 1908 ATAATAGAGCTTCAAGCAAG (SEQ ID NO:18)
1760 ATAATCGACGTTCAAGCAAG (SEQ ID NO:2)
1631 CGCGCGCGCGCGCGCGCG (SEQ ID NO:59)
1835 TCTCCCAGCGAGCGCCAT (SEQ ID NO:60)
1759 ATAATCCAGCTTGAACCAAG (SEQ ID NO:61)
20 1826 TCCATGACGTTCTGACGTT (SEQ ID NO:62)
1585 GGGGTCAACGTTGAGGGGGG (SEQ ID NO:63)
2010 GCGGCGGGCGGCGCGCGCCC (SEQ ID NO:54)
1972 GGGGTCTGTGCTTTGGGGGG (SEQ ID NO:64)
2001 GGCGGCGGCAGCGGGCGGCGG (SEQ ID NO:65)

25 Mice were pretreated with the indicated oligonucleotide and then challenged through the airways with LPS as described above. A saline challenge was used as a control. The lungs of the mice were lavaged, and the number of cell per ml, number of

- 28 -

polymorphonuclear cells (PMN) per ml, and the percentage of polymorphonuclear cells in the airways was determined (see Table 1).

Table 1**expt 1, C3H/BFeJ mice**

	<u>ODN Number</u>	<u>cells/ml x 10⁴</u>	<u>PMN/ml x 10⁵</u>	<u>% PMN</u>
5	1908	61.4±15.9	59.2±15.7	95.8±0.95
	1760	27.8±3.5*	25.8±3.0*	93.3±2.3
	1631	47.6±11.1	46.1±10.7	96.8±1.11
	1835	43.8±7.1	44.4±7.1	96.8±0.75
10	1759	71.0±19.8	67.7±20.4	96.8±2.6
	1826	39.8±7.8	38.3±7.9	93.5±1.4
	None (saline)	71.0±7.4	69.3±6.9	97.8±1.3

expt 2, C57 BL/6 mice

	<u>ODN Number</u>	<u>cells/ml x 10⁴</u>	<u>PMN/ml x 10⁵</u>	<u>% PMN</u>
15	1908	18.0±2.6	16.6±2.7	91.2±3.7
	1760	10.2±2.3*	8.6±2.1*	82.0±3.0
	1585	11.0±2.2*	9.5±2.2*	84.6±2.9
	2010	14.1±2.1	11.8±1.9	83.4±2.1
	None (saline)	17.9±3.4	16.9±2.1	

20 expt 3

	<u>ODN Number</u>	<u>cells/ml x 10⁴</u>	<u>PMN/ml x 10⁵</u>	<u>% PMN</u>
	1908	19.0±2.5	16.9±2.1	89.4±1.4
	1760	9.1±0.8*	7.7±0.7*	84.6±0.5
	1972	15.3±1.6	13.5±1.4	84.2±1.2
25	2001	13.0±1.6*	11.8±1.6	90.4±2.2

* P < 0.05, Mann-Whitney U test

- 29 -

In experiment 1, both oligonucleotide 1760 and 1826 appear to be effective. In experiment 2, oligonucleotide 1760 and 1585 were effective. Oligonucleotide 2010 also appears to have had a modest effect. In experiment 3, oligonucleotide 1760 was effective. A modest effect was seen with oligonucleotide 2001. Thus the best 5 oligonucleotides for inducing a therapeutic effect fit the motifs shown in SEQ ID NO:1 and SEQ ID NO:3. Oligonucleotides such as 2001 and 2010, which contain CpG motifs with CCGG, CCG, and CGG, can also have a beneficial effect.

The results indicate that CpG containing oligonucleotides substantially reduce the inflammatory response to inhaled LPS and that the protective effect appears to be 10 specific to unmethylated CpG motifs embedded within the oligonucleotide. These findings suggest that oligonucleotides containing CpG motifs may prove helpful in controlling the inflammatory response to inhaled LPS and other environmental agents.

EXAMPLE 3

THE ROLE OF IL-10

15 Since endogenous and exogenous IL-10 are known to suppress the inflammatory response to LPS (Cassatella, M.A., *et al.*, *J. Exp. Med.* 178:2207, 1993; Berg, D.J., *et al.*, *J. Clin. Invest.* 96:2339-2347, 1995), IL-10 might play a critical role in mediating the immunosuppressive effects of CpG oligonucleotides. To pursue this hypothesis, IL-10 knockout (C57BL/6-IL10^{tm1Cgn}) mice and C57BL/6 control mice were pretreated with 20 CpG containing oligonucleotides and then an inhalation challenge with *E. coli* LPS was performed. Compared to pretreatment with intravenous saline, CpG containing oligonucleotides significantly reduced the total cellularity and the concentration of PMNs in the lavage fluid in both C57BL/6 and mice with a disrupted IL-10 gene (C57BL/6-IL10^{tm1Cgn}) (FIG. 6). Importantly, the immunosuppressive effects of CpG oligonucleotides 25 were equally effective in mice with a disrupted IL-10 gene compared to wild type mice.

The results indicate that the protective effect of unmethylated CpG motifs is not dependent on IL-10.

- 30 -

EXAMPLE 4

INDUCTION OF NK ACTIVITY

Phosphodiester ODN were purchased from Operon Technologies (Alameda, CA). Phosphorothioate ODN were purchased from the DNA core facility, University of Iowa, 5 or from The Midland Certified Reagent Company (Midland TX). *E. coli* (strain B) DNA and calf thymus DNA were purchased from Sigma (St. Louis, MO). All DNA and ODN were purified by extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and/or ethanol precipitation. The LPS level in ODN was less than 12.5 ng/mg and *E. coli* and calf thymus DNA contained less than 2.5 ng of LPS/mg of DNA by Limulus assay.

10 Virus-free, 4-6 week old, DBA/2, C57BL/6 (B6) and congenitally athymic BALB/C mice were obtained on contract through the Veterans Affairs from the National Cancer Institute (Bethesda, MD). C57BL/6 SCID mice were bred in the SPF barrier facility at the University of Iowa Animal Care Unit.

15 Human peripheral blood mononuclear leukocytes (PBMC) were obtained as previously described (e.g., Ballas, Z.K. *et al.*, *J. Allergy Clin. Immunol.* 85:453, 1990). Human or murine cells were cultured at 5×10^6 /well, at 37°C in a 5% CO₂ humidified atmosphere in 24-well plates with medium alone or with CpG or non-CpG ODN at the indicated concentrations, or with *E. coli* or calf thymus (50 µg/ml) at 37°C for 24 hr. All cultures were harvested at 18 hr. and the cells were used as effectors in a standard 4 hr.

20 ⁵¹Cr-release assay against K562 (human) or YAC-1 (mouse) target cells as previously described. For calculation of lytic units (LU), 1 LU was defined as the number of cells needed to effect 30% specific lysis. Where indicated, neutralizing antibodies against IFN-γ (Lee Biomolecular, San Diego, CA) or IL-12 (Pharmingen) or their isotype controls were added at the initiation of cultures to a concentration of 10 µg/ml. For 25 anti-IL-12 addition, 10 µg of each of the 4 MAB (or isotype controls) were added simultaneously. Recombinant human IL-2 was used at a concentration of 100 U/ml.

Experiments were conducted to determine whether CpG containing oligonucleotides

- 31 -

stimulated the activity of natural killer (NK) cells in addition to B cells. As shown in Table 2, a marked induction of NK activity among mouse spleen cells cultured with CpG ODN 1: **GCTAGACGTTAGCGT** (SEQ ID NO:19) and 3Dd: **GAGAAXGCTGGACCTTCCAT** (SEQ ID NO:20), 5 (where X = 5 methyl cytosine) was observed. In contrast, there was relatively no induction in effectors that had been treated with non-CpG control ODN.

Table 2
Induction Of NK Activity By CpG Oligodeoxynucleotides (ODN)

	ODN	% YAC-1 Specific Lysis*		% 2C11 Specific Lysis	
		Effector: Target	50:1 100:1	Effector: Target	50:1 100:1
10	None		-1.1 -1.4		15.3 16.6
	1		16.1 24.5		38.7 47.2
	3Dd		17.1 27.0		37.0 40.0
	non-CpG ODN		-1.6 -1.7		14.8 15.4

Induction of NK activity by DNA containing CpG motifs, but not by non-CpG

15 DNA

Bacterial DNA cultured for 18 hrs. at 37°C and then assayed for killing of K562 (human) or Yac-1 (mouse) target cells induced NK lytic activity in both mouse spleen cells depleted of B cells, and human PBMC, but vertebrate DNA did not (Table 3). To determine whether the stimulatory activity of bacterial DNA may be a 20 consequence of its increased level of unmethylated CpG dinucleotides, the activating properties of more than 50 synthetic ODN containing unmethylated, methylated, or no CpG dinucleotides was tested. The results, summarized in Table 3, demonstrate that synthetic ODN can stimulate significant NK activity, as long as they contain at least one unmethylated CpG dinucleotide (Ballas, Z., et al., *J Immunol* **157**:1840-1845, 25 1996). No difference was observed in the stimulatory effects of ODN in which the CpG was within a palindrome (such as ODN 1585, which contains the palindrome

- 32 -

AACGTT) from those ODN without palindromes (such as 1613 or 1619), with the caveat that optimal stimulation was generally seen with ODN in which the CpG was flanked by two 5' purines or a 5' GpT dinucleotide and two 3' pyrimidines. Kinetic experiments demonstrated that NK activity peaked around 18 hrs. after addition of the 5 ODN. The data indicates that the murine NK response is dependent on the prior activation of monocytes by CpG DNA, leading to the production of IL-12, TNF- α , and IFN.

Table 3

Induction of NK Activity by DNA Containing CpG Motifs but not by Non-CpG DNA

			LU/10 ⁶	
		DNA or Cytokine Added	Mouse	Cells
10	Human Cells	Expt. 1	None	0.00 0.00
			IL-2	16.68 15.82
			E. Coli DNA	7.23 5.05
			Calf thymus DNA	0.00 0.00
15		Expt. 2	None	0.00 3.28
			1585 ggGGTCAAC <u>CG</u> TTGACgggg (SEQ ID NO:21)	7.38 17.98
			1629 -----gtc----- (SEQ ID NO:22)	0.00 4.4
20		Expt. 3	None	0.00
			1613 GCTAGAC <u>CG</u> TTAGTGT	(SEQ ID NO:23) 5.22
			1769 -----X-----	(SEQ ID NO:24) 0.02 ND
			1619 TCCATGTC <u>CG</u> TTCCCTGATGCT (SEQ ID NO:5)	3.35
25			1765 -----X-----	(SEQ ID NO:25) 0.11

- 33 -

CpG dinucleotides in ODN sequences are indicated by underlining; X indicates methylcytosine. Lower case letters indicate nuclease resistant phosphorothioate modified internucleotide linkages which, in titration experiments, were more than 20 times as potent as non-modified ODN, depending on the flanking bases. Poly G ends (g) were 5 used in some ODN, because they significantly increase the level of ODN uptake. Dashes indicate some bases are identical to those in the directly preceding sequence, with the exception of changes noted.

Immune activation by CpG motifs may depend on bases flanking the CpG, and the 10 number and spacing of the CpGs present within an ODN. Although a single CpG in an ideal base context can be a very strong and useful immune activator, superior effects can be seen with ODN containing several CpGs with the appropriate spacing and flanking bases. For activation of murine B cells, the optimal CpG motif is TGACGTT.

The following studies were conducted to identify optimal ODN sequences for stimulation 15 of human cells by examining the effects of changing the number, spacing, and flanking bases of CpG dinucleotides.

Identification of phosphorothioate ODN with optimal CpG motifs for activation of human NK cells

To have clinical utility, ODN must be administered to a subject in a form that protects 20 them against nuclease degradation. Methods to accomplish this with phosphodiester ODN are well known in the art and include encapsulation in lipids or delivery systems such as nanoparticles. This protection can also be achieved using chemical substitutions to the DNA such as modified DNA backbones including those in which the internucleotide linkages are nuclease resistant. Some modifications may confer additional desirable properties such as increasing cellular uptake. For example, the 25 phosphodiester linkage can be modified via replacement of one of the nonbridging oxygen atoms with a sulfur, which constitutes phosphorothioate DNA. Phosphorothioate ODN have enhanced cellular uptake (Krieg *et al.*, *Antisense Res. Dev.* 6:133, 1996) and

improved B cell stimulation if they also have a CpG motif. Since NK activation correlates strongly with *in vivo* adjuvant effects, the identification of phosphorothioate ODN that will activate human NK cells is very important.

The effects of different phosphorothioate ODNs, which contain CpG dinucleotides in 5 various base contexts, on human NK activation (Table 4) were examined. ODN 1840, which contained 2 copies of the TGTCGTT motif, had significant NK lytic activity (Table 4). To further identify additional ODNs optimal for NK activation, approximately one hundred ODN containing different numbers and spacing of CpG motifs, were tested with ODN 1982 serving as a control. Sample results are shown in Table 5.

10 Effective ODNs generally began with a TC or TG at the 5' end, however, this requirement was not mandatory. ODNs with internal CpG motifs (e.g., ODN 1840) are generally less potent stimulators than those in which a GTCGCT motif immediately follows the 5' TC (e.g., ODN 1967 and 1968). ODN 1968, which has a second GTCGTT motif in its 3' half, was consistently more stimulatory than ODN 1967, which lacks this 15 second motif. ODN 1967, however, was slightly more potent than ODN 1968 in experiments 1 and 3, but not in experiment 2. ODN 2005, which has a third GTCGTT motif, induced slightly higher NK activity on average than 1968. However, ODN 2006, in which the spacing between the GTCGTT motifs was increased by the addition of two 20 Ts between each motif, was slightly superior to ODN 2005 and to ODN 2007, in which only one of the motifs had the addition of the spacing two Ts. The minimal acceptable spacing between CpG motifs is one nucleotide as long as the ODN has two pyrimidines (preferably T) at the 3' end (e.g., ODN 2015). Surprisingly, joining two GTCGTT motifs end to end with a 5' T also created a reasonably strong inducer of NK activity (e.g., ODN 2016). The choice of thymine (T) separating consecutive CpG dinucleotides 25 is not absolute, since ODN 2002 induced appreciable NK activation despite the fact that adenine (a) separated its CpGs (i.e., CGACGTT). It should also be noted that ODNs containing no CpG (e.g., ODN 1982), runs of CpGs, or CpGs in bad sequence contexts (e.g., ODN 2010) had little or no stimulatory effect on NK activation.

- 35 -

Table 4
ODN induction of NK Lytic Activity (LU)

ODN	Sequence (5'-3')	LU
None		0.01
5 1754	ACCATGGACGATCTGTTCCCCTC (SEQ ID NO:26)	0.02
1758	TCTCCCAGCGTGCGCCAT (SEQ ID NO:27)	0.05
1761	TACCGCGTGCACCCCTCT (SEQ ID NO:28)	0.05
1776	ACCATGGACGAACTGTTCCCCTC (SEQ ID NO:29)	0.03
1777	ACCATGGACGAGCTGTTCCCCTC (SEQ ID NO:30)	0.05
10 1778	ACCATGGACGACCTGTTCCCCTC (SEQ ID NO:31)	0.01
1779	ACCATGGACGTACTGTTCCCCTC (SEQ ID NO:32)	0.02
1780	ACCATGGACGGTCTGTTCCCCTC (SEQ ID NO:33)	0.29
1781	ACCATGGACGTTCTGTTCCCCTC (SEQ ID NO:34)	0.38
1823	GCATGACGTTGAGCT (SEQ ID NO:35)	0.08
15 1824	CACGTTGAGGGGCAT (SEQ ID NO:36)	0.01
1825	CTGCTGAGACTGGAG (SEQ ID NO:37)	0.01
1828	TCAGCGTGCGCC (SEQ ID NO:38)	0.01
1829	ATGACGTTCTGACGTT (SEQ ID NO:39)	0.42
1830 ²	RANDOM SEQUENCE	0.25
20 1834	TCTCCCAGCGGGCGCAT (SEQ ID NO:40)	0.00
1836	TCTCCCAGCGCGCGCCAT (SEQ ID NO:41)	0.46
1840	TCCATGTCGTTCTGTCGTT (SEQ ID NO:42)	2.70
1841	TCCATAGCGTTCTAGCGTT (SEQ ID NO:43)	1.45
1842	TCGTCGCTGTCTCCGCTTCTT (SEQ ID NO:44)	0.06
25 1851	TCCTGACGTTCTGACGTT (SEQ ID NO:45)	2.32

¹Lytic units (LU) were measured as described (8). Briefly, PBMC were collected from normal donors and spun over Ficoll, then cultured with or without the indicated ODN (which were added to cultures at 6 µg/ml) for 24 hr. Then their ability to lyse ⁵¹Cr-labeled K562 cells was determined. The results shown are typical of those obtained with several different normal human donors.

²This oligo mixture contained a random selection of all 4 bases at each position.

Table 5
Induction of NK LU by Phosphorothioate CpG ODN with Good Motifs

ODN ¹	sequence (5'-3')	expt. 1	expt. 2	expt. 3
None		0.00	1.26	0.46
5 1840	TCCATGTCGTTCCCTGTCGTT (SEQ ID NO:42)	2.33	ND	ND
1960	TCCTGTCGTTCCCTGTCGTT (SEQ ID NO:46)	ND	0.48	8.99
1961	TCCATGTCGTTCCCTGTCGTT (SEQ ID NO:47)	4.03	1.23	5.08
1962	TCCTGTCGTTCCCTGTCGTT (SEQ ID NO:13)	ND	1.60	5.74
1963	TCCTGTCGTTCCCTGTCGTT (SEQ ID NO:48)	3.42	ND	ND
10 1965	TCCTGTCGTTCCCTGTCGTT (SEQ ID NO:14)	0.46	0.42	3.48
1966	TCGTCGCTGTCCTCCGCTTCTT (SEQ ID NO:44)	2.62	ND	ND
1967	TCGTCGCTGTCCTCCGCTTCTT (SEQ ID NO:15)	5.82	1.64	8.32
1968	TCGTCGCTGTCCTCCGCTTCTT (SEQ ID NO:16)	3.77	5.26	6.12
1979 ²	TCCATGTCGTTCCCTGTCGTT (SEQ ID NO:49)	1.32	ND	ND
15 1982	TCCAGGACTCTCTCTCAGGTT (SEQ ID NO:50)	0.05	ND	0.98
1990	TCCATGCGTGCCTGCCTT (SEQ ID NO:51)	2.10	ND	ND
1991	TCCATGCGTGCCTGCCTT (SEQ ID NO:52)	0.89	ND	ND
1992	TCCACGACCGTTTCGACGTT (SEQ ID NO:53)	4.02	1.31	9.79
2002	TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:7)	ND	4.22	12.75
2005	TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:6)	ND	6.17	12.82
2006	TCGTCGTTGTCGTTGTCGTT (SEQ ID NO:8)	ND	2.68	9.66
2007	TGTCGTTGTCGTTGTCGTT (SEQ ID NO:10)	ND	1.37	8.15
2008	GCGTGCCTGTCGTTGTCGTT (SEQ ID NO:54)	ND	0.01	0.05
2010	GCGGCGGGGGGGGGGCC (SEQ ID NO:54)	ND	2.02	11.61
2012	TGTCGTTGTCGTTGTCGTT (SEQ ID NO:11)	ND	5.74	10.89
25 2013	TGTCGTTGTCGTTGTCGTT (SEQ ID NO:55)	ND	4.53	10.13
2014	TGTCGTTGTCGTTGTCGTT (SEQ ID NO:12)	ND	6.54	8.06
2015	TGTCGTTGTCGTT (SEQ ID NO:56)	ND		
2016	TGTCGTTGTCGTT (SEQ ID NO:56)	ND		

¹PBMC essentially as described herein. Results are representative of 6 separate experiments; each experiment represents a different donor.

²This is the methylated version of ODN 1840; Z=5-methyl cytosine LU is lytic units; ND = not done; CpG dinucleotides are underlined for clarity.

5

EXAMPLE 5

IDENTIFICATION OF PHOSPHOROTHIOATE ODN WITH OPTIMAL CPG MOTIFS FOR ACTIVATION OF HUMAN B CELL PROLIFERATION

The ability of a CpG ODN to induce B cell proliferation is a good measure of its adjuvant potential. Indeed, ODN with strong adjuvant effects in mouse studies also induce B cell proliferation. To determine whether the optimal CpG ODN for inducing B cell proliferation are the same as those for inducing NK cell activity, similar panels of ODN (Table 6) were tested. Many CpG ODN were stimulatory. ODN 2006 produced the most consistent stimulation (Table 6).

Table 6
Induction of human B cell proliferation by Phosphorothioate CpG ODN

DN	sequence (5'-3') ¹	Stimulation Index ¹					
		expt. 1	expt. 2	expt. 3	expt. 4	expt. 5	expt. 6
1840	TCCAT <u>GTCGTTCCCTGTCGTT</u> (SEQ ID NO:42)	4	ND	ND	ND	ND	ND
1841	TCCATAG <u>CGTTCCCTAGCGTT</u> (SEQ ID NO:43)	3	ND	ND	ND	ND	ND
1960	TCC <u>GTGCGTTCCCTGTCGTT</u> (SEQ ID NO:46)	ND	2.0	2.0	3.6	ND	ND
1961	TCC <u>ATGTCGTTTCGTCGTT</u> (SEQ ID NO:47)	2	3.9	1.9	3.7	ND	37
1962	TCC <u>TGTCGTTCCCTGTCGTT</u> (SEQ ID NO:13)	ND	ND	3.8	1.9	3.9	5.4
1963	TCC <u>TGTCGTTCCCTGTCGTT</u> (SEQ ID NO:48)	3	ND	ND	ND	ND	ND
1965	TCC <u>TGTCGTTTCGTCGTT</u> (SEQ ID NO:14)	4	3.7	2.4	4.7	6.0	43
1967	TC <u>GTCGCTGTCGCTCTCT</u> (SEQ ID NO:15)	ND	4.4	2.0	4.5	5.0	36
1968	TC <u>GTGCGCTGTCGTTCTCT</u> (SEQ ID NO:16)	ND	4.0	2.0	4.9	8.7	38
1982	TCC <u>AGGACTCTCTCAAGGTT</u> (SEQ ID NO:50)	3	1.8	1.3	3.1	3.2	12
2002	TCC <u>ACGTTTCGACGTT</u> (SEQ ID NO:53)	ND	2.7	1.4	4.4	ND	14

2005	<u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:7)	5	3.2	1.2	3.0	7.9	37
2006	<u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:6)	4	4.5	2.2	5.8	8.3	40
2007	<u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:8)	3	4.0	4.2	4.1	ND	22
2008	<u>G</u> <u>C</u> <u>G</u> <u>T</u> <u>G</u> <u>C</u> <u>G</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:9)	ND	3.0	2.4	1.6	ND	12
2010	<u>G</u> <u>C</u> <u>G</u> <u>C</u> <u>G</u> <u>C</u> <u>G</u> <u>C</u> <u>G</u> <u>C</u> <u>G</u> <u>C</u> <u>C</u> <u>C</u>	(SEQ ID NO:54)	ND	1.6	1.9	3.2	ND	ND
2012	<u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:10)	2	2.8	0	3.2	ND	33
2013	<u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:55)	3	2.3	3.1	2.8	ND	7
2014	<u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:11)	3	2.5	4.0	3.2	6.7	14
2015	<u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:12)	5	1.8	2.6	4.5	9.4	1
2016	<u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u> <u>G</u> <u>T</u> <u>C</u> <u>G</u> <u>T</u> <u>T</u>	(SEQ ID NO:56)	ND	1.1	1.7	2.7	7.3	1

'Cells = human spleen cells stored at -70°C after surgical harvest or PBMC collected from normal donors and spun over Ficoll. Cells were cultured in 96 well U-bottom microtiter plates with or without the indicated ODN (which were added to cultures at 6 μ M). N = 12 experiments. Cells were cultured for 4-7 days, pulsed with 1 μ Ci of 3 H thymidine for 18 hr before harvest and scintillation counting. Stimulation index = the ratio of cpm in wells without ODN to that in wells that had been stimulated throughout the culture period with the indicated ODN (there were no further additions of ODN after the cultures were set up). ND = not done

- 40 -

EXAMPLE 6
IDENTIFICATION OF PHOSPHOROTHIOATE ODN
THAT INDUCE HUMAN IL-12 SECRETION

The ability of a CpG ODN to induce IL-12 secretion is a good measure of its adjuvant potential, especially in terms of its ability to induce a Th1 immune response, which is highly dependent on IL-12. Therefore, the ability of a panel of phosphorothioate ODN to induce IL-12 secretion from human PBMC *in vitro* (Table 7) was examined. These experiments showed that in some human PBMC, most CpG ODN could induce IL-12 secretion (e.g., expt. 1). However, other donors responded to just a few CpG ODN (e.g., expt. 2). ODN 2006 was a consistent inducer of IL12 secretion from most subjects (Table 7).

Table 7
Induction of human IL-12 secretion by Phosphorothioate CpG ODN

	ODN _i	sequence (5'-3')	IL-12 (pg/ml)	
			expt. 1	expt. 2
15	None		0	0
	1962	TCCTGTCGTTCCCTTGTGCGTT (SEQ ID NO:13)	19	0
	1965	TCCTGTCGTTTTGTGCGTT (SEQ ID NO:14)	36	0
	1967	TCGTCGCTGTCCTGCCCTTCTT (SEQ ID NO:15)	41	0
	1968	TCGTCGCTGTTGTGCGTTCTT (SEQ ID NO:16)	24	0
20	2005	TCGTCGTTGTCGTTGTGCGTT (SEQ ID NO:7)	25	0
	2006	TCGTCGTTTGTCGTTTGTCGTT (SEQ ID NO:6)	29	15
	2014	TGTCGTTGTCGTTGTGCGTT (SEQ ID NO:11)	28	0
	2015	TCGTCGTCGTCGTT (SEQ ID NO:12)	14	0
	2016	TGTCGTTGTCGTT (SEQ ID NO:57)	3	0

25 ¹PBMC were collected from normal donors and spun over Ficoll, then cultured at 10⁶ cells/well in 96 well microtiter plates with or without the indicated ODN which were added to cultures at 6 µg/ml. Supernatants were collected at 24 hr and tested for IL-12 levels by ELISA as described in methods. A standard curve was run in each experiment, which represents a different donor.

- 41 -

EXAMPLE 7
IDENTIFICATION OF B CELL AND
MONOCYTE/NK CELL-SPECIFIC OLIGONUCLEOTIDES

CpG DNA can directly activate highly purified B cells and monocytic cells. There are
5 many similarities in the mechanism through which CpG DNA activates these cell types.
For example, both require NFkB activation as explained further below.

In further studies of different immune effects of CpG DNA, it was found that there is
more than one type of CpG motif. Specifically, oligo 1668, with the best mouse B cell
motif, is a strong inducer of both B cell and natural killer (NK) cell activation, while
10 oligo 1758 is a weak B cell activator, but still induces excellent NK responses (Table 8).

Table 8
Different CpG motifs stimulate optimal murine B cell and
NK activation

	ODN	Sequence	B cell activation ⁱ	NK activation ⁱ
15	1668	TCCATGAC <u>CG</u> TTCCCTGATGCT (SEQ ID NO:57)	42,849	2.52
	1758	TCTCCCAG <u>CG</u> TGC <u>GC</u> CAT (SEQ ID NO:27)	1,747	6.66
	NONE		367	0.00

CpG dinucleotides are underlined; oligonucleotides were synthesized with phosphorothioate modified backbones to improve their nuclease resistance.

20 ⁱMeasured by ³H thymidine incorporation after 48 hr culture with oligodeoxynucleotides at a 200 nM concentration as described in Example 1.

ⁱMeasured in lytic units.

- 42 -

EXAMPLE 8

PREVENTION OF THE DEVELOPMENT OF AN INFLAMMATORY
CELLULAR
INFILTRATE AND EOSINOPHILIA IN A MURINE MODEL OF ASTHMA

- 5 Six to eight week old C56BL/6 mice (from The Jackson Laboratory, Bar Harbor, ME) were immunized with 5,000 *Schistosoma mansoni* eggs by intraperitoneal (i.p.) injection on days 0 and 7. *Schistosoma mansoni* eggs contain an antigen (*Schistosoma mansoni* egg antigen (SEA)) that induces a Th2 immune response (e.g., production of IgE antibody). IgE antibody production is known to be an important cause of asthma.
- 10 The immunized mice were then treated with oligonucleotides (30 μ g in 200 μ l saline by i.p. injection), which either contained an unmethylated CpG motif, i.e.,
TCCATGACGTTCCCTGACGTT (SEQ ID NO:39),
or did not, i.e., control,
TCCATGAGCTTCCTGAGTCT (SEQ ID NO:58).
- 15 Soluble SEA (10 μ g in 25 μ l of saline) was administered by intranasal instillation on days 14 and 21. Saline was used as a control.

Mice were sacrificed at various times after airway challenge. Whole lung lavage was performed to harvest airway and alveolar inflammatory cells. Cytokine levels were measured from lavage fluid by ELISA. RNA was isolated from whole lung for Northern analysis and RT-PCR studies using CsCl gradients. Lungs were inflated and perfused with 4% paraformaldehyde for histologic examination.

FIG. 7 shows that when the mice are initially injected with the eggs i.p., and then inhale the egg antigen (open circle), many inflammatory cells are present in the lungs. However, when the mice are initially given a nucleic acid containing an unmethylated CpG motif along with the eggs, the inflammatory cells in the lung are not increased by subsequent inhalation of the egg antigen (open triangles).

FIG. 8 shows that the same results are obtained when only eosinophils present in the lung lavage are measured. Eosinophils are the type of inflammatory cell most closely associated with asthma.

- 43 -

FIG. 9 shows that when the mice are treated with a control oligo at the time of the initial exposure to the egg, there is little effect on the subsequent influx of eosinophils into the lungs after inhalation of SEA. Thus, when mice inhale the eggs on days 14 or 21, they develop an acute inflammatory response in the lungs. However, giving a CpG oligo 5 along with the eggs at the time of initial antigen exposure on days 0 and 7 almost completely abolishes the increase in eosinophils when the mice inhale the egg antigen on day 14.

FIG. 10 shows that very low doses of oligonucleotide (< 10 μ g) can give this protection.

FIG. 11 shows that the resultant inflammatory response correlates with the levels of the 10 Th2 cytokine IL-4 in the lung.

FIG. 12 shows that administration of an oligonucleotide containing an unmethylated CpG motif can actually redirect the cytokine response of the lung to production of IL-12, indicating a Th1 type of immune response.

FIG. 13 shows that administration of an oligonucleotide containing an unmethylated CpG 15 motif can also redirect the cytokine response of the lung to production of IFN- γ , indicating a Th1 type of immune response.

What is claimed is:

- 44 -

1. A method of treating a subject having or at risk of having an acute decrement in air flow, comprising:
administering to a subject a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG.
2. The method of claim 1, wherein the nucleic acid sequence is from 8-30 bases in length.
3. The method of claim 1, wherein the subject is human.
4. The method of claim 1, wherein the acute decrement in airflow results from lipopolysaccharide (LPS) exposure.
5. The method of claim 1, wherein the nucleic acid sequence has a formula:
$$5'N_1X_1CGX_2N_23' \text{ (SEQ ID NO:1)}$$
wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
6. The method of claim 5, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.
7. The method of claim 5, wherein said nucleic acid sequence is SEQ ID NO:2.
8. The method of claim 1, wherein the nucleic acid sequence has a formula:
$$5'N_1X_1X_2CGX_3X_4N_23' \text{ (SEQ ID NO:3)}$$
wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
9. The method of claim 8, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

- 45 -

10. A method of inhibiting an inflammatory response in a subject having inhaled or at risk of having inhaling lipopolysaccharide (LPS), comprising:
administering to a subject a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG.
11. The method of claim 10, wherein the nucleic acid sequence is from 8-30 bases in length.
12. The method of claim 10, wherein the subject is human.
13. The method of claim 10, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1CGX_2N_23'$ (SEQ ID NO:1)
wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
14. The method of claim 13, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.
15. The method of claim 13, wherein said nucleic acid sequence is SEQ ID NO:2.
16. The method of claim 10, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1X_2CGX_3X_4N_23'$ (SEQ ID NO:3)
wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
17. The method of claim 16, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

- 46 -

18. A method of modifying the level of a cytokine in a subject having inhaled or at risk of inhaling lipopolysaccharide (LPS), comprising:
administering to a subject a therapeutically effective amount of a nucleic acid sequence containing at least one unmethylated CpG dinucleotide.
19. The method of claim 18, wherein the nucleic acid sequence is from 8-30 bases in length.
20. The method of claim 18, wherein the subject is human.
21. The method of claim 18, wherein said nucleic acid sequence is SEQ ID NO:2.
22. The method of claim 18, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1CGX_2N_23'$ (SEQ ID NO:1)
wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
23. The method of claim 22, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.
24. The method of claim 18, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1X_2CGX_3X_4N_23'$ (SEQ ID NO:3)
wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases
25. The method of claim 24, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.
26. The method of claim 18, wherein said modulation is a reduction in the level of said cytokine.

- 47 -

27. The method of claim 18, wherein said modulation is an increase in the level of said cytokine.
28. The method of claim 18, wherein said cytokine is selected from the group consisting of TNF- α , MIP-2, IL-10, IL-12, and interferon- γ .
29. A pharmaceutical composition for treating a subject having or at risk of having an acute decrement in airflow, comprising:
a nucleic acid sequence containing at least one unmethylated CpG in a pharmacologically acceptable carrier.
30. The composition of claim 29, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1CGX_2N_23'$ (SEQ ID NO:1)
wherein at least one nucleotide separates consecutive CpGs; X_1 is adenine, guanine, or thymidine; X_2 is cytosine or thymine, N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
31. The composition of claim 30, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.
32. The composition of claim 30, wherein said nucleic acid SEQ ID NO:2.
33. The composition of claim 29, wherein the nucleic acid sequence has a formula:
 $5'N_1X_1X_2CGX_3X_4N_23'$ (SEQ ID NO:3)
wherein at least one nucleotide separates consecutive CpGs; X_1X_2 is selected from the group consisting of GpT, GpG, GpA, ApT and ApA; X_3X_4 is selected from the group consisting of TpT or CpT; N is any nucleotide and $N_1 + N_2$ is from about 0-26 bases.
34. The composition of claim 33, wherein N_1 and N_2 do not contain a CCGG quadmer or more than one CCG or CGG trimer; and the nucleic acid sequence is from about 8-30 bases in length.

- 48 -

35. An isolated nucleic acid sequence comprising at least one unmethylated CpG dinucleotide having a sequence as set forth in a member of the group consisting of SEQ ID NO:2, SEQ ID NO: 17, SEQ ID NO:18, SEQ ID NO: 59, SEQ ID NO: 60, SEQ ID NO: 61, SEQ ID NO:62, SEQ ID NO: 63, SEQ ID NO:64 and SEQ ID NO:65.

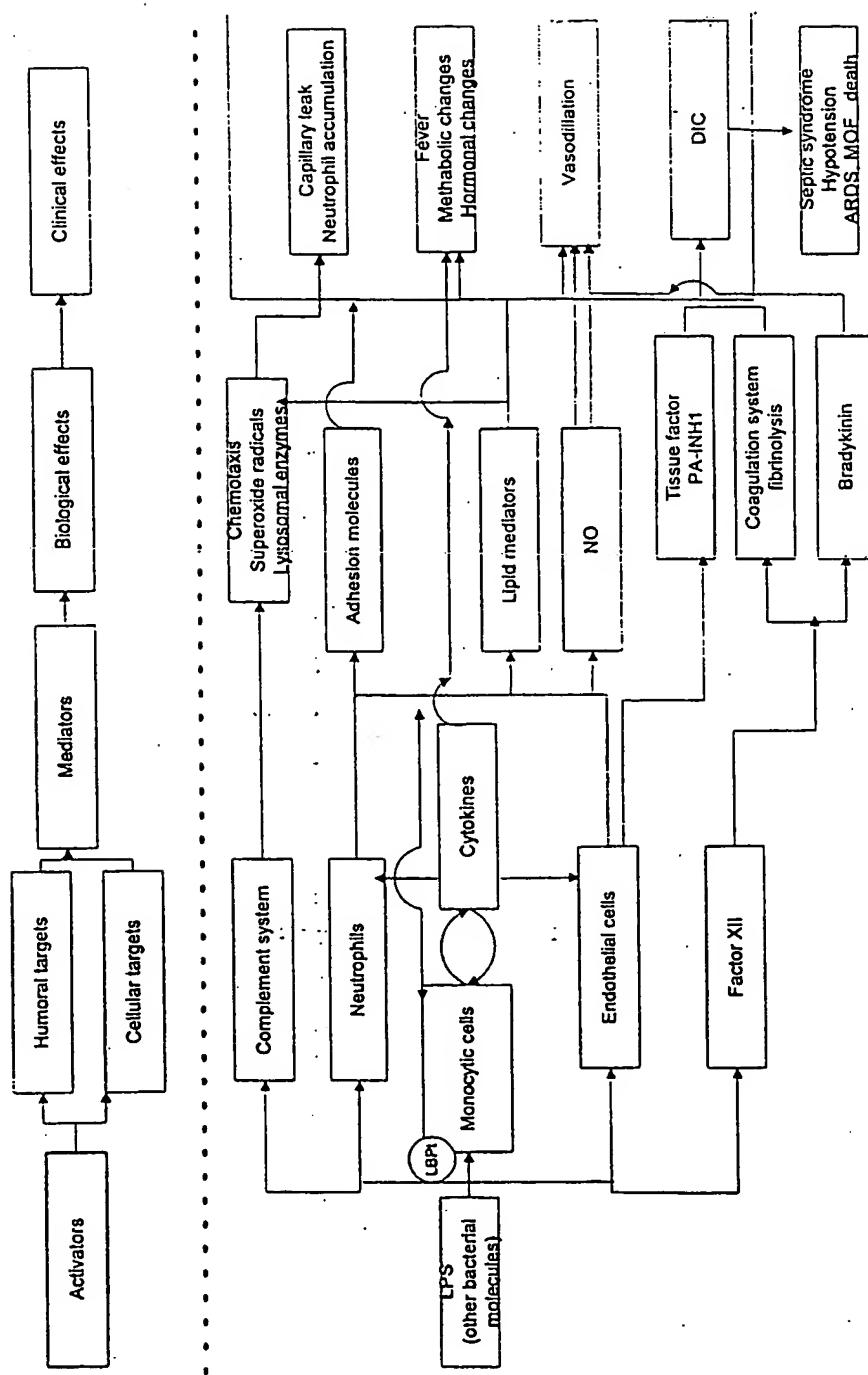
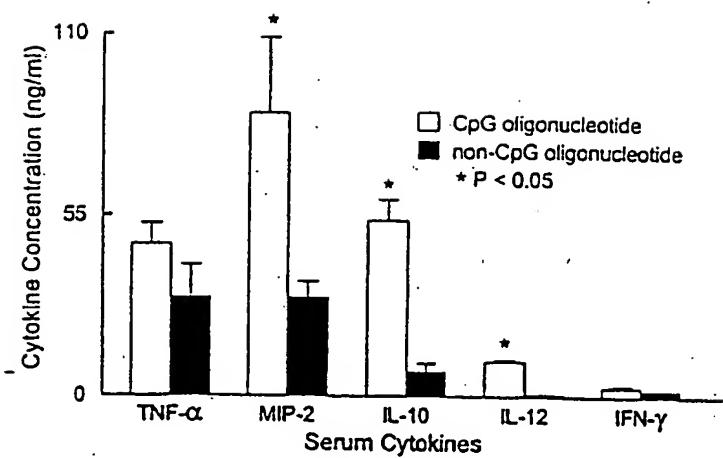


Fig. 1

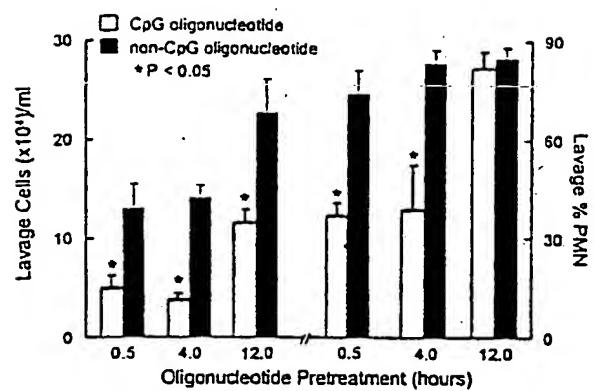
2/14

FIGURE 2.



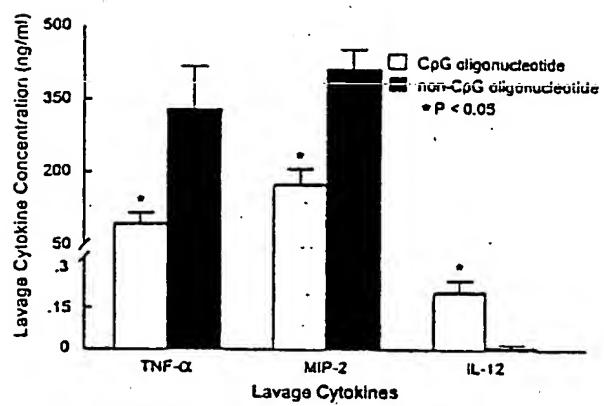
3/14

FIGURE 3



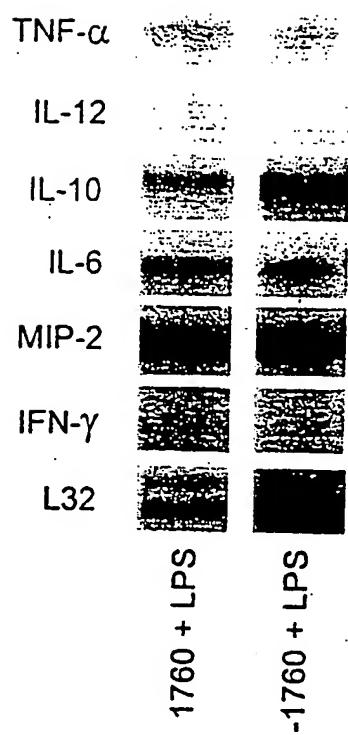
4/14

FIGURE 4



5/14

FIGURE 5



6/14

FIGURE 6

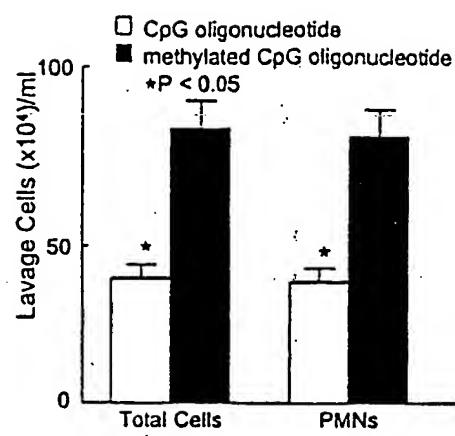
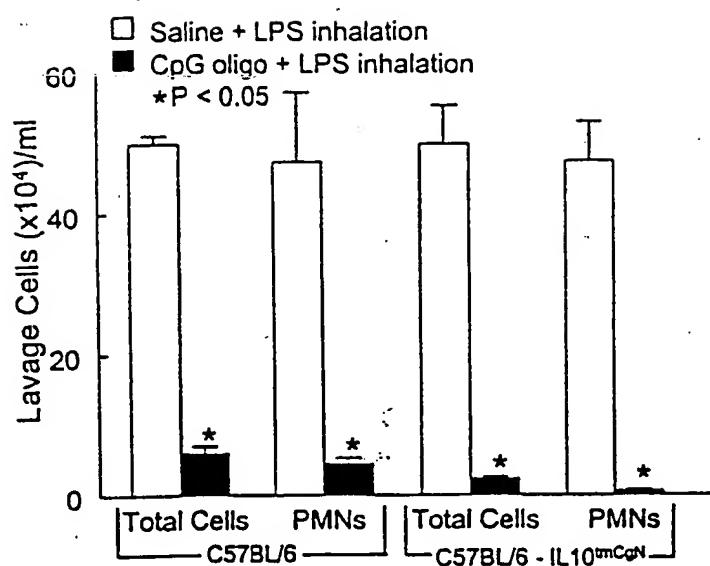


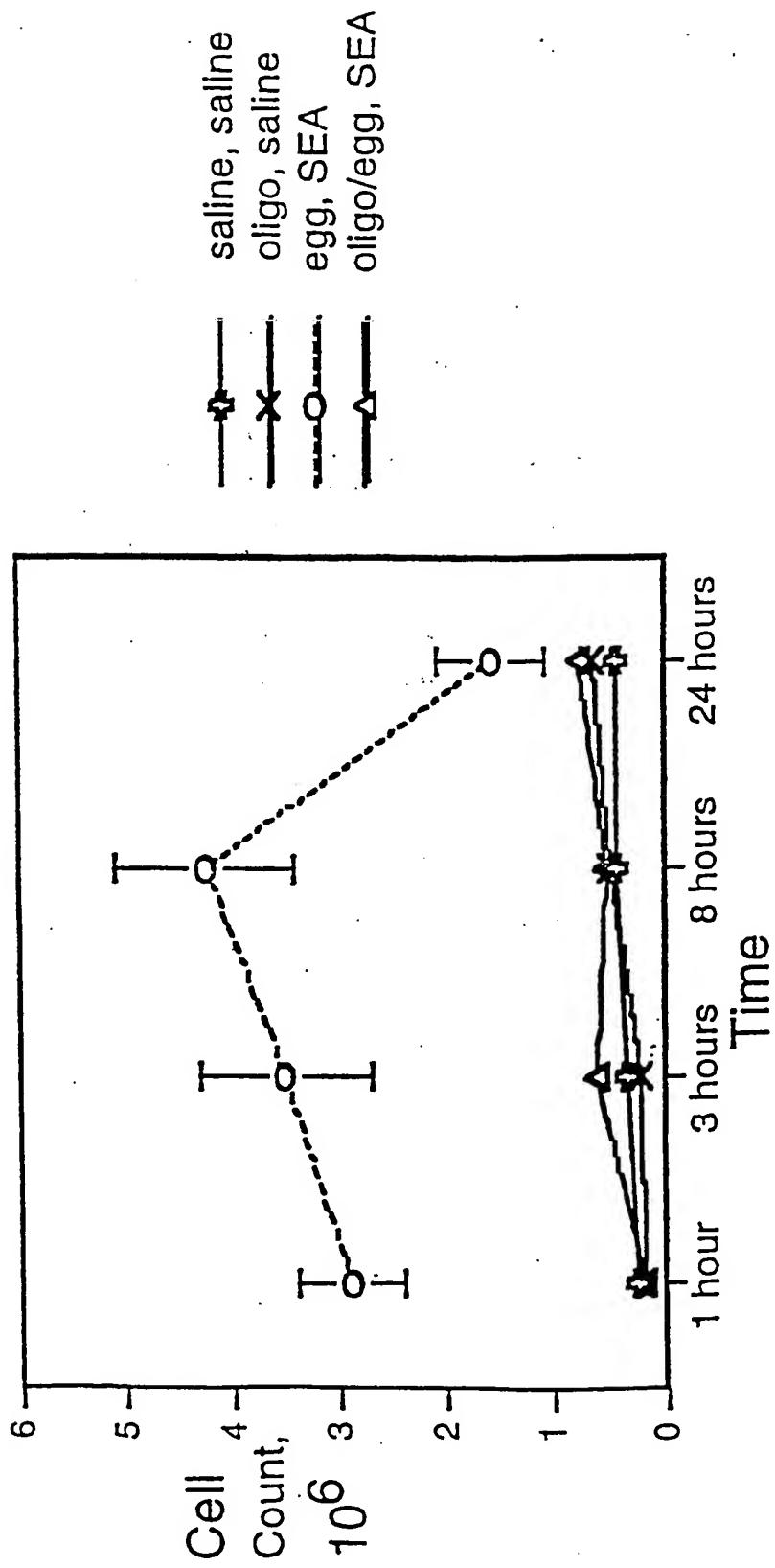
FIGURE 7



8/14

Effect of CpG and Airway Exposure on Lung Lavage Cell Count

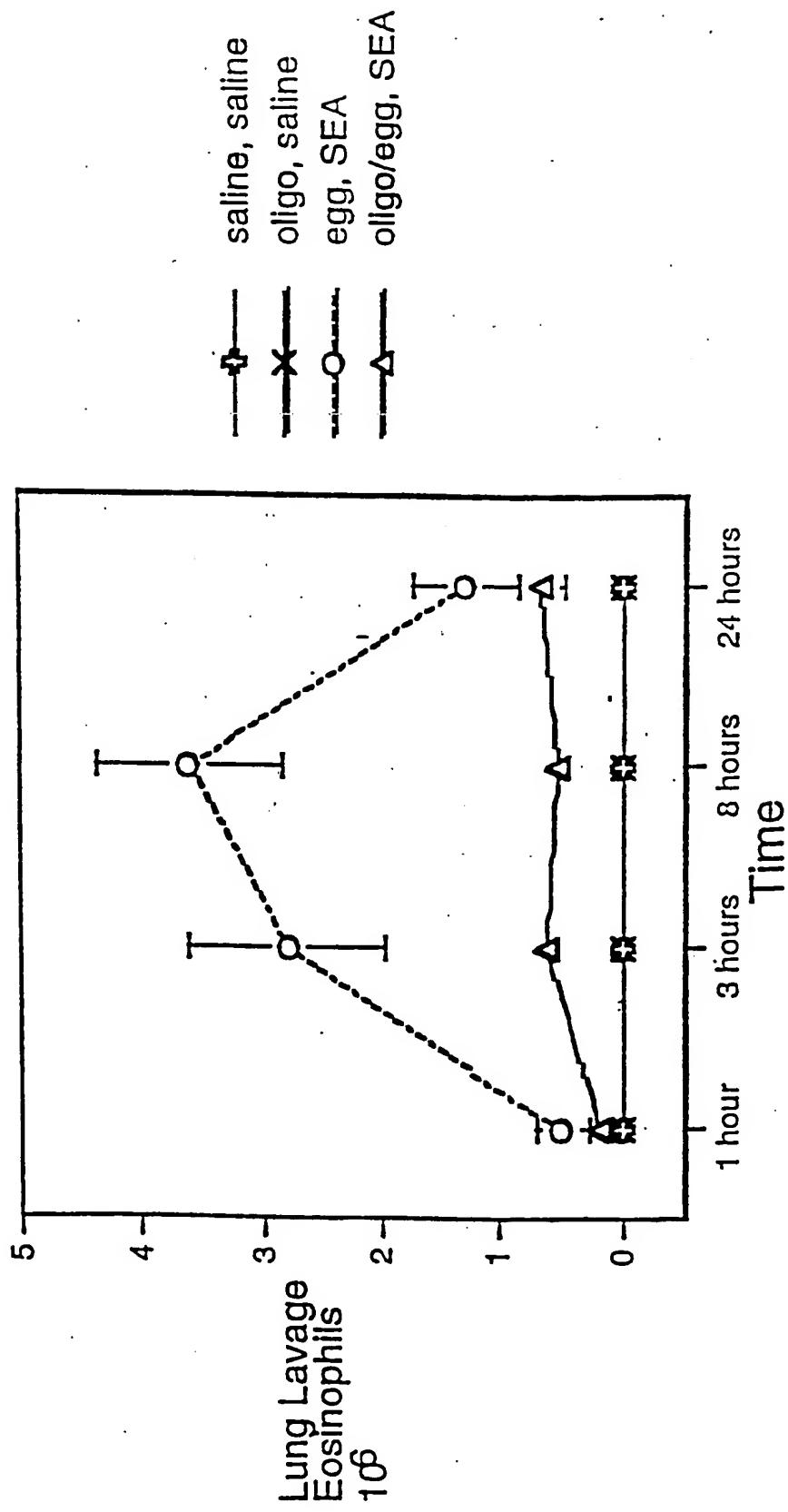
FIGURE 8



9/14

Effect of CpG and Airway Exposure On Lung Lavage Eosinophil Count

FIGURE 9



Effect of CpG and Airway Exposure on Lung Lavage Differential

FIGURE 10

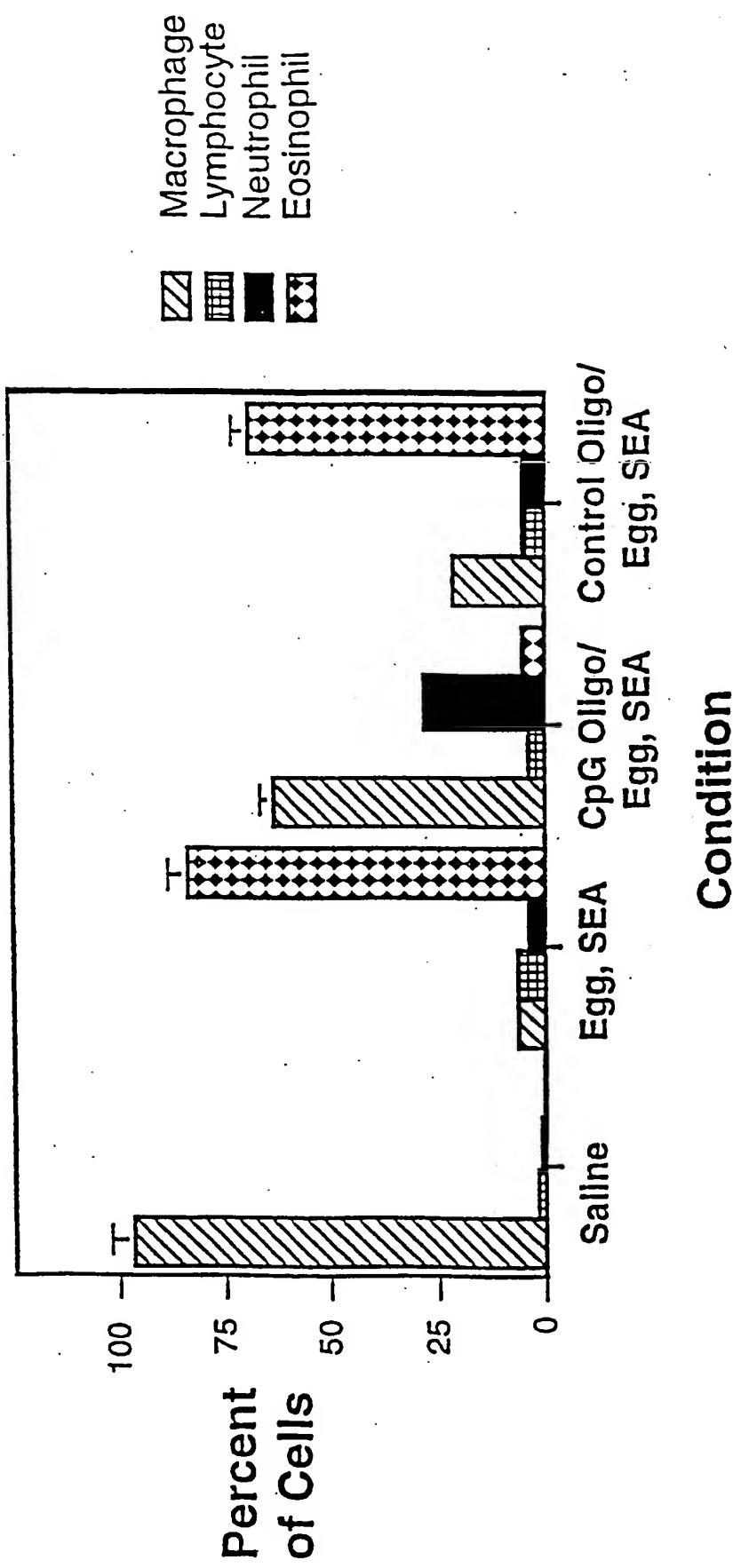
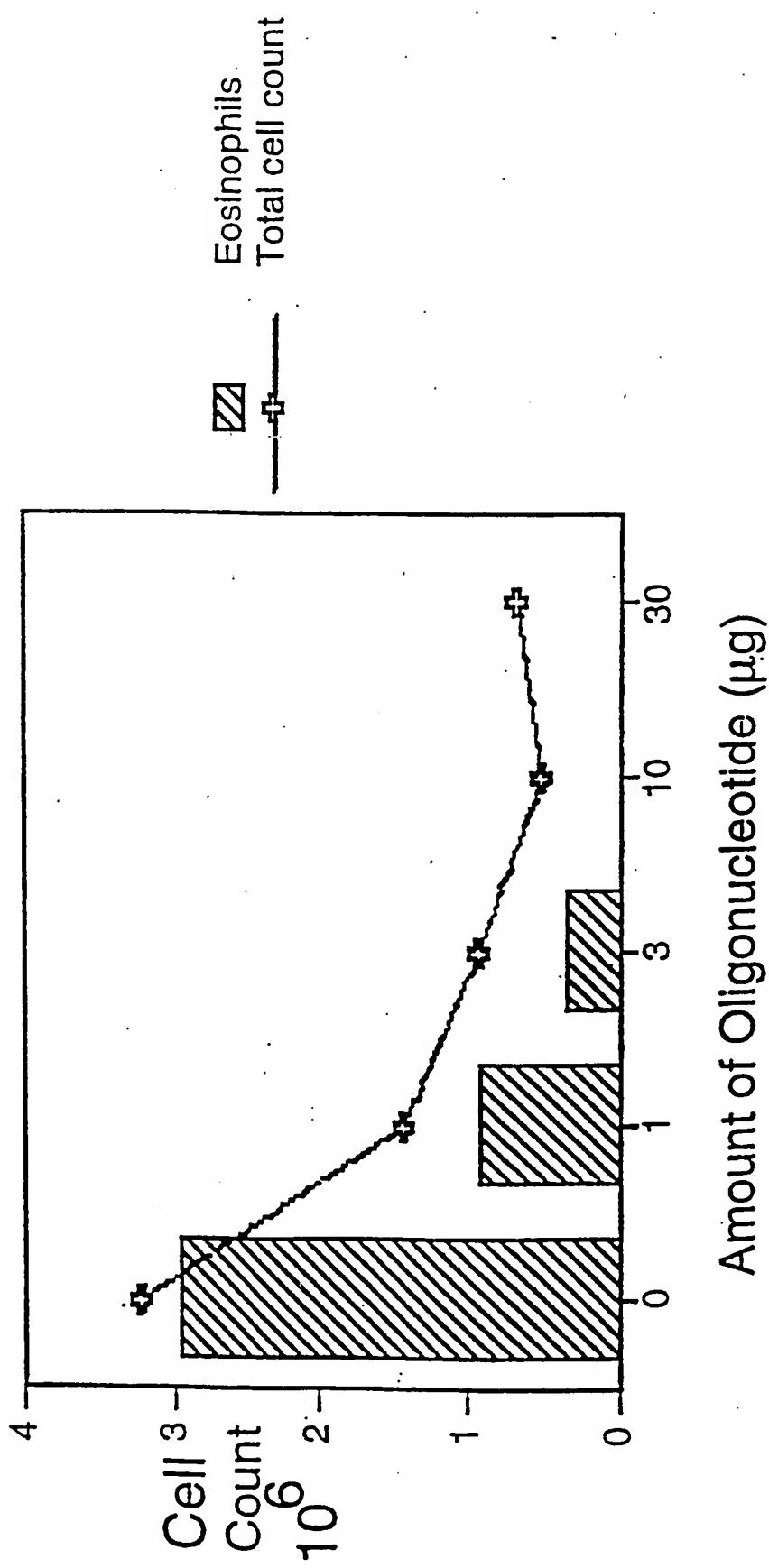


FIGURE 11

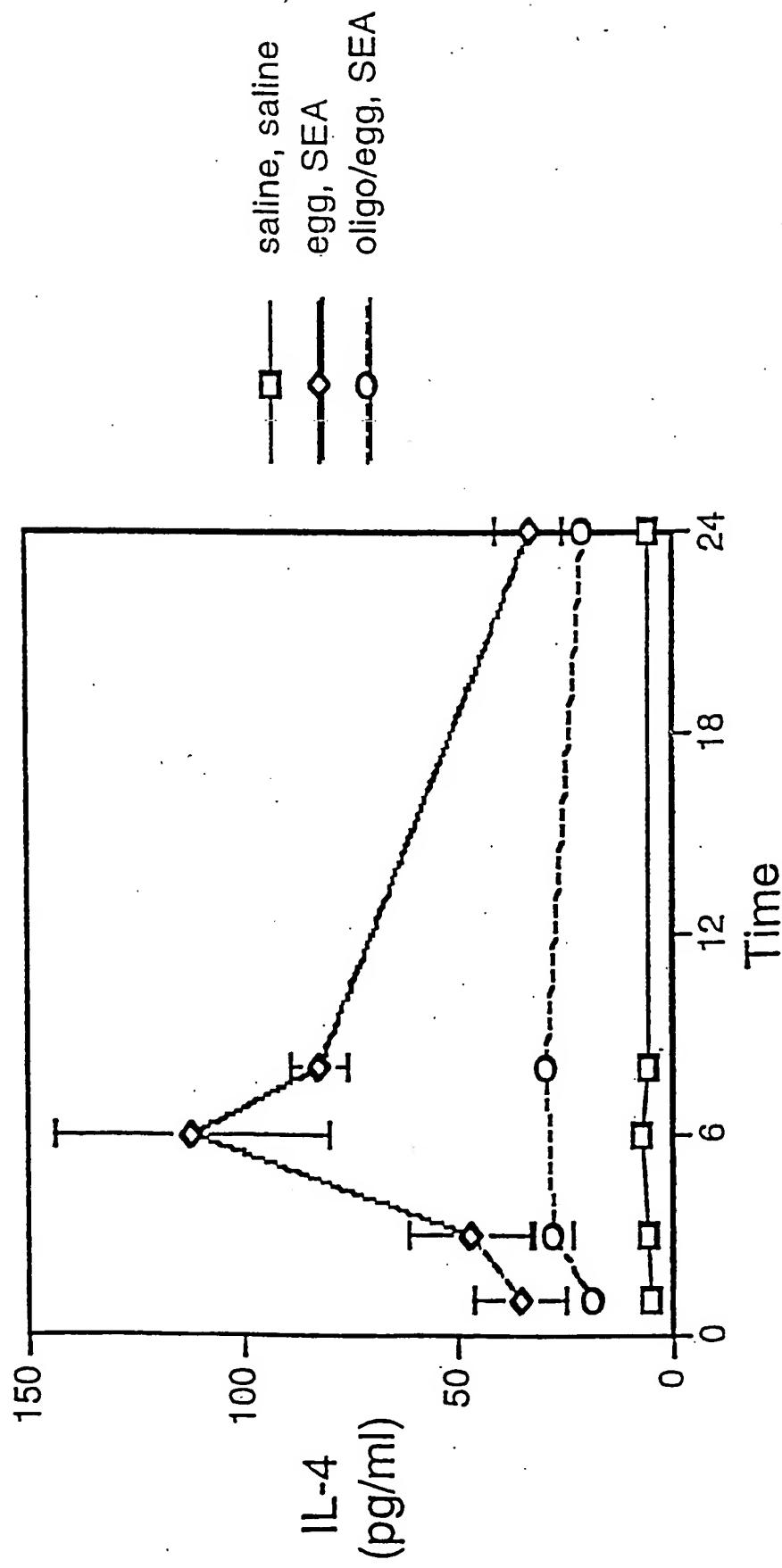
Effect of Oligonucleotide Dose on Total and Eosinophil Cell Counts



12/14

FIGURE 12

Effect of CpG and Airway Exposure on Lung Lavage IL-4



13/14

FIGURE 13

Effect of CpG and Airway Exposure on Lung Lavage IL-12

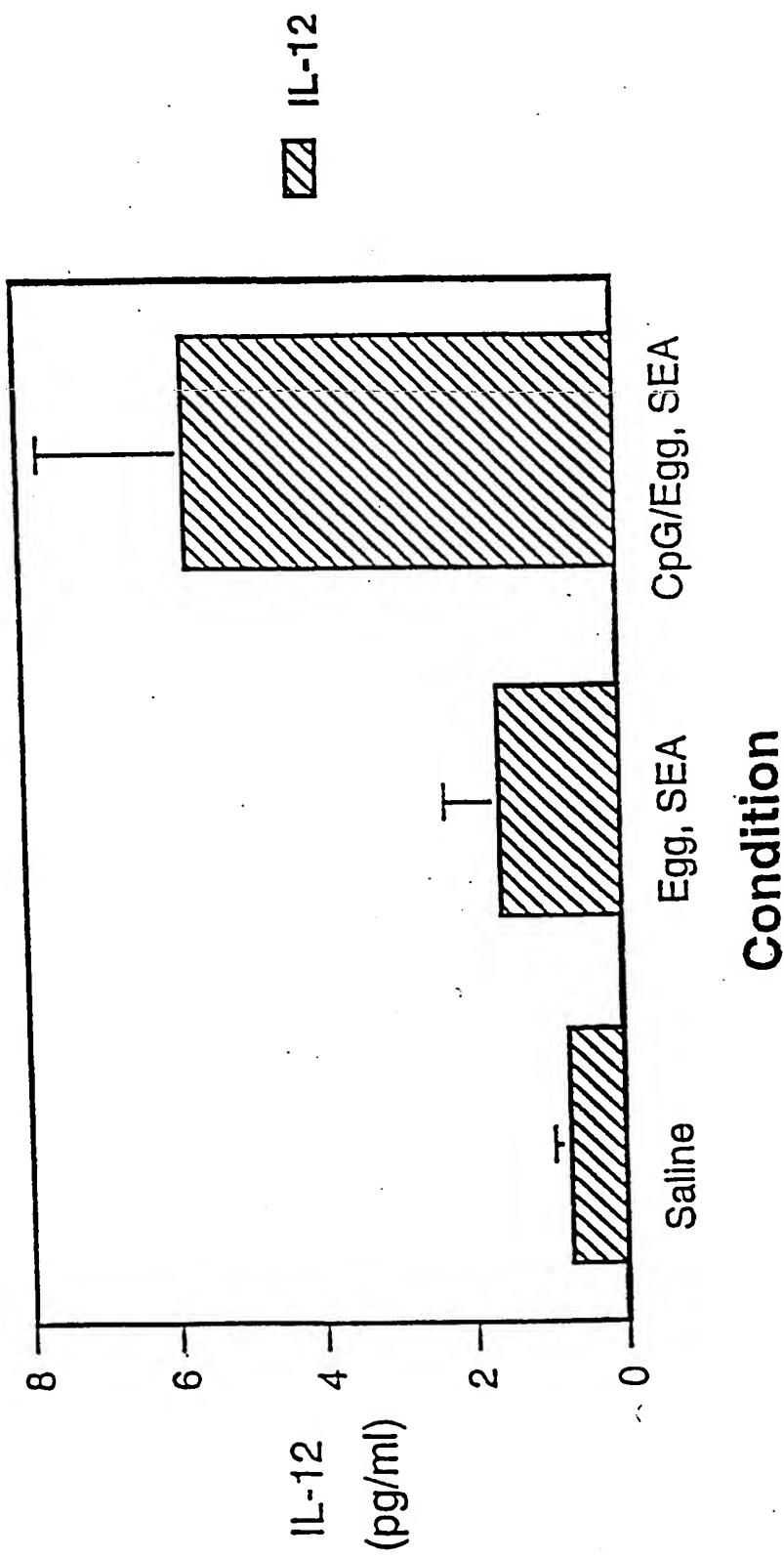
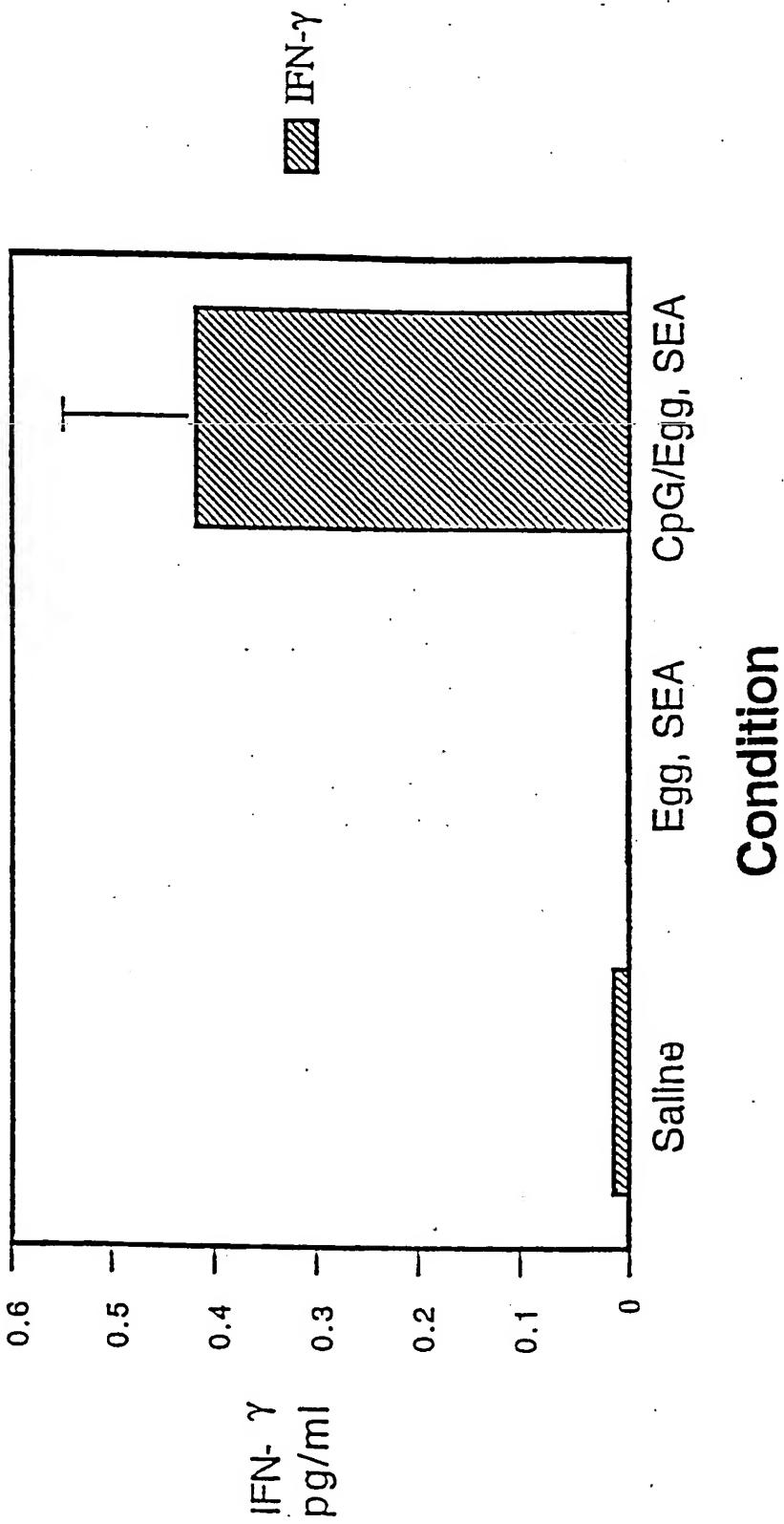


FIGURE 14

Effect of CpG and Airway Exposure on Lung Lavage IFN- γ



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03678

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 49/00; C07H 21/02, 21/04; A01N 43/04
US CL : 536/23.1, 24.5; 514/44; 424/9.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 24.5; 514/44; 424/9.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, SCISEARCH, EMBASE, WPIDS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YI et al. IFN- γ Promotes IL-6 and IgM Secretion in response to CpG Motifs in Bacterial DNA and Oligodeoxynucleotides. J. Immunology. 15 January 1996, Vol. 156, No. 2, pages 558-564, see entire document.	29-34 -----
Y	BLACKMAN et al. Specific 5' and 3' regions of the μ -chain gene are undermethylated at distinct stages of B-cell differentiation. Proc. Natl. Acad. Sci. June 1985, Vol. 82, pages 3809-3813, see entire document.	1-28
X	THREADGILL et al. Mitogenic synthetic polynucleotides suppress the antibody response to a bacterial polysaccharide. Vaccine. January 1998, Vol. 16, No. 1, pages 76-82, see entire document.	29-34
X,P		1-34

 Further documents are listed in the continuation of Box C. See patent family annex.

Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"A"	document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

30 APRIL 1998

Date of mailing of the international search report

23 JUN 1998

Name and mailing address of the ISA/US

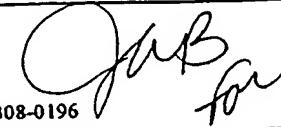
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ANDREW WANG

Telephone No. (703) 308-0196



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/03678

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRANDA et al. Amplification of antibody production by phosphorothioate oligodeoxynucleotides. J. Lab. Clin. Med. September 1996, Vol. 128, No. 3, pages 329-338, see entire document.	29-34
---		-----
Y		1-28